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Million-dollar problem—billion-dollar solution?

THE OECD report on the transport of air pollutants which Dr Barnes summarises on pages 92–93 is bound to resurrect some political problems that have been held just below the surface whilst the report was being prepared. The most important is likely to be the question of whether the United Kingdom should assume any responsibility for the effects of acid rainfall in Norway.

In the past thirty years the greatly increased use of tall smoke stacks by industry has been a blessing to those who live in industrial areas and who are no longer bathed in the acidic fumes of sulphur dioxide and sulphates. But the disagreeable material is now not simply dispersed over a wider area surrounding the smoke stack; much of it resides for a long time in the mixing layer that extends to a height of about 1,200 metres and which has been described as a travelling garbage can. The material is eventually either dry-deposited or brought down to the earth as acidic precipitation.

The OECD study reports sulphur deposition levels in rural areas in 1974 and attempts, by an understanding of prevailing winds and precipitation, to estimate how much of the material comes from the country in question, and how much from elsewhere. A table attempts to summarise the export–import budget for most European countries (including estimates for Eastern Europe). It is open to misinterpretation by politicians who do not appreciate the very large error bars (at least $\pm 50\%$) that must be applied to the data and the relatively short period of reporting (one year).

Even so, some interesting figures emerge. In many countries exports and imports of sulphur roughly balance, but Austria, Finland, Norway and Sweden and Switzerland each involuntarily import more than twice what they export. Denmark and the United Kingdom, on the other hand, export much more sulphur than they import.

The United Kingdom is the largest sulphur-emitter in Europe, putting 1.8 million tonnes into the atmosphere in 1974 (remember, however, that the figures must not be read too precisely). Less than half of this comes back to earth in the UK—most of the rest of Europe getting a dose of British sulphur. In the case of, say, West Germany, the dose is relatively small compared with what West Germany administers to herself. But Norway, where the clouds that pass over Britain often

deposit their rain, receives roughly a quarter of her total sulphur deposit from British sources, double the contribution she makes from her own smokestacks.

This in itself by no means makes Norway into an exceptionally polluted part of the world—wet deposition of sulphates in southern Norway runs at about the same level as in much of the rest of Europe. But a lot of this sulphate resides in snow for several months and is the first to melt when temperatures rise in spring. The acid flush down rivers (pH 3 to 4) then kills fish, doing damage to the value of around \$1 million. Sulphur also does other damage, of course, through corrosion and possibly by affecting agricultural productivity (though this is not yet well understood) but such damage is probably common to many countries whereas the fish kill problem is mainly relevant to Norway. It has also to be said that Norway worries about its declining environment in much the way the United Kingdom worries about its declining economy.

But now we know a little better what happens to sulphur, what is going to happen? Norway, which in the past has arranged for journalists to be flown to see the ravages caused by British pollution, will presumably go on the offensive again and demand better removal of SO_2 and sulphates at source. The United Kingdom will say that the study represents only one year and is of necessity so inexact that policy decisions cannot be based on the report.

The bleak fact is that this diseconomy, on a scale of ten million dollars at most, can only be countered by corrective action on a Europe-wide basis costing at least one thousand million dollars, maybe ten times as much. There is, of course, a solution of sorts, and that is for Britain to supply Norway with limestone to be added to lakes during the acid flush. No-one is yet sure that this will save the fish without some other side effect emerging. And of course it is of the nature of a technological fix—a now much reviled practice; but so, for that matter, would be anything attached at vast cost to chimneys. Nonetheless, the Norwegian government would be foolish to reject such a proposal out of hand—as seems quite likely. An insistence on removing pollution at source calls for so much investment and will generate so much international ill-will that any more flexible solution must first be considered. □



Twelve months after Séveso

A YEAR AGO last Sunday, Séveso 'happened'. A trichlorophenol reactor at the Icmesa chemical plant there spewed its contents over the surrounding area. They included about 1 kg of a highly toxic by-product, 2,3,7,8-tetrachlorodibenzo-*paradioxin*, known as dioxin. Controversy has raged ever since. It has involved both the plant's Swiss owners (Givaudan, a subsidiary of Hoffman-La Roche) and the Italian authorities; the scientific, legal and other aspects of the disaster; and, above all, the many people affected directly or indirectly by the dioxin. Séveso has received probably the most extensive media coverage ever accorded to the site of an industrial accident, and is a watchword for pollution.

Amidst all this two facts about the disaster are irrefutable, their consequences disastrous. The first was the venting of the reactor discharge directly to the atmosphere, an obvious fault of design. The second was the two-week delay in evacuating residents from the area, responsibility for which cannot with certainty be established but is part of the deliberations of the Italian court investigating the accident. For the people exposed to the dioxin, the health picture shows 106 children with the skin disease *chloracne*, but no evidence of immunological damage; no evidence of malformation attributable to the dioxin in the foetuses legally aborted from Séveso women exposed to the chemical, and no evidence of chromosomal damage in these foetuses; and a number of children born with abnormalities in the Séveso region which is reported to be no greater than the

statistical norm for the area.

The plan to monitor the health of everyone exposed to the dioxin, though medically and politically necessary, will not be a true epidemiological survey of 'dioxin risk'. A more selective survey of categories of 'high, low and non-risk' groups is under review and will yield results more rapidly. There are fewer grounds for optimism over the question of decontamination policy for the removal of the dioxin. The section of Séveso which housed most of the evacuated residents has been decontaminated by the plant's owners and pronounced safe for habitation. But the methods to be adopted for the remainder of the contamination area are still disputed.

What are the wider implications of Séveso? The issue of reform of the abortion laws in Italy is one. Another concerns one of the proposals of the 1976 World Health Assembly which advocated that WHO collate information on birth defects in the populations of member countries; the collation is now more urgent than ever. The lessons for the general public, the chemical industry and governments are obvious. There are dangers involved in the operation of chemical plants. And the cost of disasters in the industry is great. Governments need to keep their pollution control measures under constant review. The fact remains, however, that it takes a major disaster to make things happen; and that has been of little comfort to the people of Séveso over the past 12 months. □

Rational containment on recombinant DNA

The US National Institutes of Health has recommended that all recombinant DNA experiments, involving any animal virus, should be subject to the highest containment conditions, even though other work with the intact virus may require less stringent conditions. Lennart Philipson, of the Department of Microbiology, Uppsala University, Sweden, and Pierre Tiollais, of the Institut Pasteur, Paris, put forward the case for categorising recombinant DNA experiments involving animal viruses more rationally.

THE development of molecular genetics has depended largely on the detailed analysis of bacterial viruses, the bacteriophages, and their use to transfer bacterial genes between bacteria which do not normally recombine. Animal viruses now promise to provide a similarly detailed insight into the molecular mechanisms of gene expression in animal cells, which will undoubtedly contribute to an understanding of man and his domestic animals both in health and disease and, it is hoped, contribute to the development of medical and veterinary science.

Restriction enzyme mapping of the viral genome enables regions of interest to be excised and analysed. In many cases the full development of this work requires the use of recombinant DNA techniques to purify and clone the resulting portions of the viral genome. Animal viruses can also be used as vectors for introducing other eukaryote DNA into animal cells in which its expression may be studied. At present, however, the guidelines on recombinant DNA research laid down by the US National Institutes of Health (NIH) require almost all recombinant DNA experiments involving animal viruses to be carried out under the highest containment conditions. In practice these requirements preclude most experiments.

Animal viruses as a class are unfortunately regarded by the general public and the uninformed scientific community

as rather mysterious entities which cause pandemics, epidemics and isolated cases of dreadful infectious diseases against which there is no remedy. Scientists themselves have coloured this picture by suggesting that viruses may cause cancer in man, implying to the layman that cancer is an infectious disease. In reality, animal viruses are extremely diverse; some are known to be highly infectious and pathogenic. Others seem to be harmless. It should therefore be mandatory to discriminate between different viruses according to their pathogenicity to man and other mammals when formulating guidelines for the use of viral genomes in *in vitro* recombinant DNA research.

The conjectural hazards envisaged arise from the (hypothetical) risk that virus genomes, when introduced into bacteria or when used as vectors for introducing DNA into mammalian cells, may give rise to 'new infectious entities' or may transfer their pathogenic or tumour-producing capacity from the new host back to man or other mammals. Both types of risk depend on chains of events with low probabilities.

Against these arguments it should be considered, first, that the transfer of all or part of the viral genome to bacterial cells may not be an entirely new event. For millions of years prokaryotic organisms have been exposed to eukaryotic DNA and several microorganisms have efficient systems of transferring DNA over recombinant barriers. Second, the development of a 'new infectious entity' would probably require expression of the viral genes in the prokaryotic cell. This must have a low probability since most genes from eukaryotes and viruses, which have been transferred up till now, are not faithfully expressed as RNA or protein in bacteria¹⁻⁴. Even if the inserted viral genes are expressed the resulting risk would not be greater than that from production of viral proteins in cell cultures, since only fragments of the viral genomes will be inserted in the experiments proposed.

The conjectural hazards may therefore be confined to the transfer of viral nucleic acids from the modified bacterium to an animal or human host. This would require a chain of events, each of low probability: the bacterium containing hybrid DNA must first escape from the laboratory and then establish itself in a new host. The viral nucleic acid must then enter the cells of the new host, and there express its tumorigenic or other harmful properties. Even without considering containment we are probably discussing probabilities in the range 10^{-20} – 10^{-28} for the transfer of harmful genes into the new host.

Against this background it is obviously necessary to prohibit experiments with viruses such as Lassa fever virus or Marburg virus, which are considered to be high risk viruses in diagnostic laboratories; but it is not clear that viruses classified as low risk, such as adeno, SV40 and polyoma viruses, need such high containment. SV40 virus has already been injected accidentally together with polio vaccine into millions of human subjects without any registered harmful effects⁶, although antibodies against SV40 appear in the serum. Some adenoviruses infect humans readily and about 50–80% of the population develop antibodies to adenovirus types 1 and 2 from the age of 5–10 years⁷. Even the adeno-SV40 hybrid viruses which were originally isolated when adenovirus vaccines were developed in monkey kidney cells have been introduced into large groups of military recruits as an inactivated or live vaccine without any detrimental effects^{8,9}. Careful studies have failed to find an association between adenovirus transcripts, T antigens or other adenovirus products with tumours in humans^{10,11}.

Normal biochemical work with adeno and SV40 viruses is considered to fall into the low risk category under guidelines published by the National Cancer Institute and those from the Center for Disease Control, Atlanta, Georgia. These regulations allow the use of large amounts of virus. Milligram quantities of viral DNA are handled regularly by investigators and technical personnel in several biochemical laboratories. Provided that expression of virus DNA is prohibited in the bacterial cell, which is likely (see above), it must be more risky for personnel to handle viral DNA than to insert it in a bacterial vector. The NIH guidelines request P4 and EK2 conditions to handle partial or intact viral DNA in a prokaryotic host although as we have pointed out the risks envisaged are comparable to or lower than those encountered when working with intact virions. It is possible to claim that virions present a higher risk since as long as the protein coat is present the virion may possibly escape from the laboratory and infect humans. Thus, there seems to be a disproportionately high containment requirement for work with animal virus genomes in bacteria.

It is also difficult to understand the containment requirements for work with eukaryotic vectors. In the case of SV40 it has been established that SV40 DNA will form hybrids with cell DNA when cells are infected at high multiplicity^{12,13}. Such experiments are in essence shotgun experiments, but since no *in vitro* recombination is involved it is considered a low risk experiment requiring no containment facilities. If a similar experiment is carried out *in vitro* between DNA from the same cell and SV40 virus DNA (probably with lower efficiency), it is labelled as P4 according to the NIH guidelines. Insertion of SV40 sequences into adenovirus DNA also occurs frequently when the two are cultivated together, as exemplified by the adeno-SV40 hybrid viruses^{14,15} and new hybrids recently developed (J. Sambrook and G. Fey, personal communication). To take advantage of the natural recombination between SV40 and adenoviruses requires only a moderate risk containment facility, but the NIH guidelines absolutely forbid these experiments using recombinant DNA techniques. Here there is also a distinct discrepancy between the guidelines

and other regulations for the study of animal viruses.

Most of the confusion is probably due to the fact that natural and artificial recombinant DNA research has not been considered as a whole. Only the new *in vitro* recombinant DNA technique has formed the basis for developing the NIH guidelines, although guidelines for work with dangerous microorganisms have been in existence for a long time. Consistent rules for all recombinant DNA research are therefore needed.

A good definition of recombinant DNA research might be that suggested by the Standing Advisory Committee on Recombinant DNA of the European Molecular Biology Organisation (EMBO): recombination of DNA molecules of different biological origin by any methods that overcome generally recognised natural barriers to mating, infection and recombination, to yield molecules that can be propagated in some host cells and the subsequent studies of such recombinant DNA molecules. *In vitro* recombinant DNA research is only a subsection of this definition. Within the broader meaning of the term there are several experiments now carried out in low risk containment according to generally accepted guidelines, which must be analysed and compared with the *in vitro* techniques before any regulatory guidelines are issued. It is, for example, possible to transfer the genome of several animal viruses into the capsid of unrelated viruses^{16–19}, and thereby increase the host range of the viral genes. It is also possible to fuse animal cells, which may involve undesirable transfer of viral genomes to new hosts^{20,21}. Many of these experiments involve conjectural hazards comparable to those implied by the *in vitro* recombinant DNA technique.

But in these cases the experiments also probably carry only a low risk if they involve low risk viruses, since similar events probably occur in nature. Therefore we would like to propose that the entire area of recombinant DNA research should be re-evaluated. Experiments which are considered to involve real and proven risks should be prohibited at present. The transfer, by any method, of high risk viral genomes, potent bacterial toxins and antibiotic resistance to gonococci and streptococci fall into this category. Conjectural hazards which may or may not become real should rapidly be evaluated under secure conditions. Meanwhile *in vitro* recombinant DNA research can probably proceed in several areas including the transfer of low risk viral genomes to prokaryotic cells and the development of eukaryotic vectors from low risk viral genomes.

In closing, it should be emphasised that both mutagenisation and deletion of a multitude of genes in the laboratory have, to the best of our knowledge, never provided a selective advantage for an organism in the natural environment. Some genetic manipulation with plant cells may be an exception. It is necessary for the opponents of recombinant DNA research to provide examples of such selective advantage otherwise this debate will focus more on faith than science. □

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Sulphur deposit account

R. A. Barnes looks at an OECD study on the transport of air pollutants over long distances

THE Final Report of the Organisation for Economic Cooperation and Development (OECD) study on the long range transport of air pollutants within north-west Europe was despatched last week. It has its origins in the first European network of sites to monitor the chemical composition of precipitation which was established in Scandinavia in 1947. In the 1950s this was extended to other countries and became known as the European Air Chemistry Network. A first detailed analysis of the data, performed by Odén in 1968, showed that an area of highly acid precipitation (pH 3–4) located in the industrial heart of Europe was expanding from year to year. This finding was paralleled by the observation that rivers and lakes in southern Scandinavia were also becoming more acidic and so it was decided that a more detailed study of the origins of the acidity was required.

The Scandinavians in particular have expressed great concern that the increasing acidification of inland waters was causing the observed fall in fish populations and was also reducing forestry production. In their opinion, the sources of acid precipitation were sulphur dioxide emissions from the heavily industrialised countries of north-west Europe, particularly those from the UK. This latter view was based on the assumption that south-west winds prevailed and that the UK policy of making major industrial emissions from tall chimneys (the 'tall-stack' policy) to reduce nearby surface concentration—which affects about half the total UK emission—is particularly effective in encouraging the transport of pollutants over long distances.

The OECD programme began in 1972 with the aim of determining the relative importance of local and distant sources of sulphur compounds within European countries, special attention being paid to the question of precipitation acidity. Initially ten countries agreed to participate actively—Austria, Denmark, the Federal Republic of Germany, Finland, France, the Netherlands, Norway, Sweden, Switzerland and the United Kingdom—and in 1974 Belgium also joined. Although several surveys have already been made of air pollution fluxes and depositions within certain parts of Europe, the OECD

study is the first comprehensive investigation of air pollution movement on a continental scale. Data analysis and interpretation, together with preparation of the final report, has been the responsibility of the Norwegian Institute for Air Research.

The research programme consisted of observations based primarily on a network of sampling stations, and predictive modelling. In all, 76 ground level sites, sampling on a daily basis, were in operation at one time or another—8 in the UK and 23 in Norway. They were located, with moderate success, to represent rural conditions within a geographic region. Mean sulphur dioxide and sulphate aerosol concentrations observed at these sites ranged from about $20 \mu\text{g m}^{-3}$ and $10 \mu\text{g m}^{-3}$ respectively, close to the major source regions, to about $2 \mu\text{g m}^{-3}$ and $0.5 \mu\text{g m}^{-3}$ respectively in the remote areas of northern and western Europe. The higher of the sulphur dioxide figures would be approximately a factor of 5 less than in the source areas themselves, whereas the longer lived and slowly formed sulphate species would be more or less the same. The sulphate in precipitation data show a similar geographical distribution to the air concentrations, with values ranging from 5 to less than 1 mg l^{-1} . In some areas (for example south-west Scandinavia and Switzerland), however, where locally increased precipitation produces greater deposition than in surrounding districts, the pattern is modified. Strong acid in precipitation shows a distribution similar to that of sulphate but with notable differences in some areas. It seems to be due to both sulphuric and nitric acids in proportions of between 3:1 and 1:1.

Sector analysis

A sector analysis was performed on the surface measurements. Using 850 mbar (1,200–1,500 m altitude) trajectories they were allocated to six directional arcs and a mean value was calculated. The results show that high mean concentrations and/or depositions were not randomly oriented, but were directed towards the main areas of emission. When these emitting areas lie to the west of the receptor, concentrations are relatively low but aggregate depositions high. The low concentrations are probably a consequence of the higher wind speed and fewer stagnant, stable, conditions in

westerly regimes, while the higher depositions would be related to the greater frequency of precipitation. In most parts of western Europe, higher concentrations are usually found in sectors directed towards the south and south-east, although these do not necessarily result in particularly high annual deposition totals. Winds from such directions are generally associated with a higher frequency of stagnant and stable conditions over source areas which would lead to the high concentrations, and with limited convective or frontal rainfall, wet deposition would tend to be limited. In south-west Norway, however, orographic precipitation in southerly and south-easterly winds, coupled with high sulphate concentrations, produces annual depositions in these sections comparable to those experienced in the westerly winds where precipitation is more frequent and plentiful.

Unfortunately, the sector analysis does not, in general, give a clear indication of which sources give rise to what fraction of the concentrations in a given sector. Because of its geographic position and relative isolation, however, an estimate can be made of the mean contribution of emissions in the British Isles to deposition in Norway and the Netherlands. In addition to the average $0.8 \text{ mg SO}_4 \text{ l}^{-1}$ and $25 \mu\text{Eq l}^{-1}$ strong acid background in rainfall from Atlantic air, to which sources in North America seem to contribute, emissions in the UK and Ireland give rise to $1.0 \text{ mg SO}_4 \text{ l}^{-1}$ and $20 \mu\text{Eq l}^{-1}$ strong acid. Mean concentrations at Norwegian sites due to other emission areas range up to $8.7 \text{ mg SO}_4 \text{ l}^{-1}$ and $140 \mu\text{Eq l}^{-1}$ strong acid.

Several of the participating countries used instrumented aircraft to obtain data on above-surface concentrations of air pollutants. Flights were usually made under specific meteorological conditions and never at night or in cloud. The results confirm that under these conditions, sulphur compounds are normally transported in the lowest 2 km of the atmosphere, generally with a peak concentration a few hundred metres above ground level and a progressive decline above that height. The average mixing height under flight conditions was found to be 1,200–1,300 m, although individual vertical profiles varied considerably. The flights also showed that under certain conditions distinct plumes of pollutants existed several hundred kilometres downwind of major source areas.

Reference has frequently been made to 'episodes', when substantial amounts of wet sulphate deposition occur over a period of less than a day, without any attempt at a formal definition.

Since such occasions, on which a substantial amount of the total annual wet deposition at a site may occur on one day, are likely to be of particular ecological significance, a working definition has been formulated: episode-days at a site are those with the highest wet depositions which, when summed, make up 30% of the annual total. An area is 'highly episodic' if 30% of its total annual wet deposition occurs on less than 10% of wet days. By these definitions parts of Norway, Finland, Sweden, Switzerland and Scotland are highly episodic with respect to sulphate. Sulphate episodicity has been shown to be relatively independent of precipitation episodicity.

Mathematical modelling was employed to obtain a more complete pattern of concentration and deposition in Europe. Estimates were made for 1974 only, and are unlikely to be the same for other years, especially in the case of wet deposition. One of the primary inputs to the models was detailed sulphur dioxide emission figures for the whole of Europe. For the 11 participating countries, man-made emissions were estimated to be 9 million tonnes as sulphur in 1973. Emissions of sulphur from other European countries were calculated to be 16 Mt in 1973 from much less reliable data. The total emission of approximately 25 Mt is approximately twice that in 1950, although some countries have contributed to the increase much less than others (the UK rise between 1950 and 1973, for example, was about 30%). Natural emissions were calculated to represent less than 10% of the man-made ones over the continent as a whole.

For use in the models, the national sulphur dioxide emission totals were allocated to a 127×127 km grid, using population and industrial statistics and simple seasonal adjustments made to arrive at emission figures representative

of individual days. As a first approximation 25% of the emission in any grid element was assumed to be deposited in that element.

Two models

A Lagrangian model was used to estimate the long range transport of sulphur on a continental scale and the so-called 'Trajectory Model' was employed to estimate the effect of any one country's emissions on the concentrations and depositions in any other country. Both functioned by considering emissions and depositions in individual grid squares and air movements according to 850 mbar (1,200–1,500 m) trajectories. Unfortunately, the choice of a coarse grid (which was necessitated by the low precision of emission data and trajectories, together with limited computing facilities) has caused the very steep gradients of sulphur concentration which exist in reality to be smoothed across 127 km^2 . The models therefore only give a very general picture of concentration and deposition with a considerable degree of uncertainty on the accuracy of predicted values at any given point. The relatively coarse grid also made it difficult for one of the main objectives of the programme—the effect of local sources on concentrations and depositions—to be achieved.

The model estimates of daily mean sulphur dioxide and sulphate aerosol concentrations do not agree very well with observed data, correlation coefficients ranging from insignificant values to 0.6 and 0.4 to 0.8, respectively. The poorer correlation in the case of the shorter lived species (sulphur dioxide) reflects the greater effect of grid smoothing in this case. The correspondence between observed and calculated annual mean concentrations, however, at about 0.9 in both cases, is much better.

As would be expected, the estimated dry deposition pattern for sulphur in Europe strongly reflects the emission field with a maximum of $10 \text{ g m}^{-2} \text{ y}^{-1}$ in the major source areas and a minimum of about $0.1 \text{ g m}^{-2} \text{ y}^{-1}$ in remote regions. There is a high degree of qualitative agreement and quantitative similarity between the sulphur wet deposition fields estimated by each of the two models, and that prepared from observed data. To a certain extent, this agreement was to be expected, since all three approaches employ the same grid-smoothed precipitation map. The technique used for preparing the grid assumes zero precipitation more than 100 km from the nearest observation over land and 300 km over the sea. Thus, for grid elements in the centre of the North Sea, the precipitation is reckoned to be about 10% of that in

eastern and central England. On a fairly arbitrary basis, it has been estimated that, for this reason, the contribution of British emission to sulphur wet deposition in southern Scandinavia has been overestimated by about 20%.

A regression of observed mean sulphate concentrations in precipitation against weighted mean values of calculated aerosol sulphate concentration, used by the Trajectory Model to estimate sulphur wet deposition due to one country in any other country, has shown that a good relationship ($r=0.9$) exists for Norwegian and Finnish data. The results suggest a natural background sulphate concentration of 0.8 mg l^{-1} in precipitation, a figure confirmed by the sector analysis. This regression has been used in the budget calculations for these two countries. A similar regression on data from the remaining countries, however, shows a much wider scatter ($r=0.36$) and an implied background of 2.0 mg l^{-1} . These results are probably a reflection of the influence of local sources in countries other than Norway and Finland, and would result in a great deal of the wet deposition in a given country being unattributed to any particular source. As a compromise, a relationship using all available data ($r=0.73$) and a 'background' of 1.2 mg l^{-1} is employed for all countries except Norway and Finland.

For several reasons, therefore, the Trajectory Model has not proved capable of estimating the dry or wet deposition in any one country due to emission in any other country to an accuracy greater than $\pm 40\%$. Thus, the corresponding total (dry+wet) deposition figures are not accurate to more than $\pm 50\%$. However, the estimates of the Trajectory Model do give a useful indication of the extent and magnitude of transboundary transport of sulphur compounds. Both models indicate that, within the area as a whole, about 50% of the sulphur emitted is dry deposited and 30% wet deposited. The balance leaves the region.

The OECD study has provided a first comprehensive insight into the long distance transport of air pollutants on a continental scale. It is far from definitive, but it has confirmed that sulphur compounds travel long distances in the atmosphere over Europe, and that the air quality in any one country is measurably affected, in a very complex fashion, by emissions in another. Much more precise and detailed work is required if the phenomenon is to be fully quantified, the report itself listing a number of facets which require further investigation. These could be taken into account by an Economic Commission for Europe study, at present in a planning stage. □

Estimated budget for dry plus wet deposition of sulphur for 1974 (Unit: 10^3 tonnes of sulphur)¹

Countries	Sulphur received	Sulphur emitted
Austria	300	221
Belgium	200	499
Denmark	100	312
Federal Republic of Germany	1,250	1,964
Finland	400	274
France	1,000	1,616
The Netherlands	150	391
Norway	250	91
Sweden	500	415
Switzerland	100	76
United Kingdom	1,000	2,883 ^a
Czechoslovakia, German Democratic Republic, Italy, Poland and other areas	11,000	—

¹ Numbers are rounded to one significant figure and accurate to within about $\pm 50\%$.

^a Includes 80×10^3 tonnes of sulphur from Ireland.

USA

Five-year stretch

The USA and USSR have agreed to another five years of scientific cooperation. Colin Norman reports from Washington

WHEN former President Richard Nixon travelled to Moscow in May 1972, he came away with a clutch of agreements for Soviet-American scientific exchange programmes which symbolised the new era of detente. Since then, the warmth of detente has been chilled by disagreements over arms control and human rights issues, but the scientific exchange agreements have proceeded apace. Last week, after a cordial meeting between Soviet and American officials in Washington, the agreements were renewed for another five years.

The renewal was negotiated at a high-level meeting of scientific delegations led by Dr Frank Press, President Carter's science adviser, and Academician V. A. Kirillin, Deputy Chairman of the USSR Council of Ministers, and chairman of the State Committee of the USSR Council of Ministers for Science and Technology. Press later described the meeting as "extremely cooperative, upbeat all the way". To underline the political importance attached to the agreements, President Carter met Kirillin for a widely publicised discussion shortly after the ritual signing ceremony.

This outbreak of cordiality in the midst of Soviet-American ill-feeling lends itself to a number of interpretations; the most widely touted is that the Soviets are willing to put aside their differences to continue with exchanges from which so far they have benefited greatly, while the Americans are eager to demonstrate that the Carter Administration's stand on human rights need not jeopardise mutually beneficial arrangements between the two countries. In any case, extension of the agreements was never seriously in doubt.

In preparation for last week's meeting, however, Press commissioned a review of how the exchanges have operated so far, and as a result there will be some changes in the way the arrangements will work in future. The review was carried out by a committee of the National Academy of Sciences, headed by Richard Garwin, a respected physicist who works for IBM. The Garwin panel interviewed scores of scientists who have participated in the exchange programmes, and its

central conclusion was that the agreement should be renewed because of "the positive benefits and real interaction evident in some of the projects under the agreement, [and] the intangible and unevaluated (but widely felt) non-substantive benefits commented upon by the participants". Nevertheless, the Garwin panel pinpointed a number of problems.

The science and technology agreement is in fact only one of about a dozen exchange pacts negotiated as a result of Nixon's May 1972 Moscow summit meeting (separate agreements exist for cooperation in space, environment, energy, medicine and other areas), but it is widely regarded as the most important. It established joint programmes in such fields as chemical catalysis, physics, forestry, the application of computers to management, electrometallurgy and science policy. Some 250 American scientists have participated and the cost to the US treasury has been about \$13.2 million over the five-year period.

The Garwin panel reported that progress has been decidedly mixed. In the physics programme, for example, "no exchange or substantive interaction has taken place thus far", while as a result of exchanges in the electrometallurgy programme, "familiarity with differences in approach and perceptions in solving technical problems in the USSR has stimulated a considerable amount of rethinking of technical approaches in the US".

There have also been a number of problems common to most of the programmes. For example, the Garwin panel noted that although virtually every participant interviewed emphasised the helpfulness and warmth of individual Soviet scientists at the working level, there appear to be "layers of stifling bureaucracy which enforce a rigidity in the interaction which very much diminishes its value to the US and to the USSR". As evidence of such stifling bureaucracy, the panel cites inflexibility in travel arrangements for US scientists visiting the Soviet Union and lack of notification of the time, date, or place of arrival of Soviet visitors to the United States.

But the problems do not lie entirely on the Soviet side of the exchanges. The Garwin panel noted that US delegations are often ill-prepared before they visit the Soviet Union. There is little interaction between participants in different exchange programmes, and American scientists are seldom briefed on Soviet research and development

policies and practices.

The Garwin panel recommended that a small, permanent bureaucracy should be established in the United States government to plan and co-ordinate the exchange programmes and to prepare US scientists for their visits to the Soviet Union. According to one official involved in last week's talks, such a group is likely to be established soon, probably in the National Science Foundation.

A frequent complaint raised in the United States, particularly among conservatives, is that the flow of information from the scientific exchanges has been virtually a one-way street, from West to East. The argument is that since the Soviet Union lags behind the United States in many fields, it is eager to use the exchanges as a conduit for American technology. The Garwin panel did not specifically address that concern, though it noted in its report that participants in the programme frequently point to "significant political and cultural benefits in addition to the relatively few citations of benefits to US science and technology". Such intangible benefits should also be weighed in assessing the overall impacts of the agreements, the panel suggested.

Nevertheless, the Administration is understandably sensitive to charges that the exchange agreements are resulting in a significant drain of technical information from the United States, and according to one official familiar with the programme, greater attention will in future be paid to securing a better balance of information flow throughout the programme.

Press said that some of the concerns raised by the Garwin panel were brought up during the negotiations last week, and the Soviet participants were willing to discuss ways of smoothing future arrangements. At one point, Kirillin said that if problems arise, he and Press would communicate directly by telephone to iron them out. Asked whether human rights problems were mentioned during the talks, Press said that they were not.

For their part, the Soviets seem anxious to press on with the exchanges, while the Carter Administration seems to feel that for an outlay of only about \$2-3 million a year, the agreement is an inexpensive way to foster better relations. □

Correction

In last week's item about Czechoslovakia a line crept in to confuse the meaning of a sentence. Dr Zdenek Mlynar's dismissal was for being a member of the campaign known as Charter 77. Our apologies.

USSR

PROFESSOR Veniamin Levich was dismissed from his post at Moscow University in 1972 as a result of his application for a visa to emigrate to Israel. He had done definitive work in a number of fields including electrochemistry and physicochemical hydrodynamics. A number of Soviet scientists working in related fields were invited to attend a conference in Oxford to celebrate his 60th birthday. All declined. Extensive extracts from a letter explaining the Soviet attitude, and from a reply from Sir Derek Barton, Chairman of the Conference Committee, and Professor D. B. Spalding, Conference Organiser, appear below

Dear Colleagues,—Over the past few months many of us have received invitations to an international conference in mid-July on "Physical Chemistry and Hydrodynamics" organised in connection with the 60th birthday of Professor V. G. Levich, corresponding-member of the USSR Academy of Sciences. For a number of reasons that are outlined below we had not intended to reply to the invitations. However, since our silence could have been misunderstood and misinterpreted, we decided to explain our attitude in this letter. . . .

You will agree that it is most unusual for a scientific conference marking the 60th birthday of a Soviet scientist, a corresponding-member of our Academy of Sciences, to have been organised and to be held in another country, without any prior consultation or agreement with organisations representing Soviet science. There is no denying that there are many major scientists in our country whose work has received universal recognition in the world. A number of them have won the Nobel Prize. But none, to our knowledge, has won such attention as Professor V. G. Levich. That leads us to believe that in this case it was not Professor Levich's scientific services that prompted this attention. Scientific services alone would not warrant it. Therefore, there must be some other reason. Apparently, it is because over the past few years Professor Levich has been busy besmirching his country, clearly to the detriment of his scientific work.

Dear Colleagues,—We have been gratified by the interest which you have shown in the Levich Conference. Other Soviet scientists have written in answer to the invitations; some have been friendly, and others less so; but none have set out their views at such length. Your courtesy is much appreciated. . . .

. . . We remark with pleasure that recognition of Professor Levich's contributions to science is not something which divides us.

Concerning paragraph 3 [2 above], we do agree that the holding of this conference in Oxford rather than Moscow is somewhat unusual. Had the USSR Academy of Sciences announced that it intended to celebrate the occasion suitably, the Oxford Conference might not have been convened. Were there any such intentions? Our enquiries revealed none.

We do not know how to compare the attention accorded to the Levich Conference with those celebrations which other scientists have enjoyed; but it is gratifying to the Conference secretariat to know that their publicising efforts have been successful. Our own belief is that the popularity of the conference has much to do with the high importance of physico-chemical hydrodynamics. This subject is, for many scientists, inevitably

We cannot, naturally, exclude the possibility that many of the participants in the conference, and perhaps its organisers as well, honestly think there has been prejudice in Professor Levich's treatment by the leaders of his Institute, of the Academy of Sciences and perhaps also other bodies in our country. Maybe that prompted them to take such an unusual step in what they regard as support or defence of Professor Levich. But even if that were the case, it would be interesting to know their source of information.

Why, when inviting us to the conference, did nobody ask for our opinion? Why was it decided to accept implicitly the information received from Professor Levich, while the views of those who worked with him and who could provide a more objective assessment were ignored? We, consequently, feel justified in concluding that our point of view does not interest the organisers of the conference, that they are not interested in the truth of the matter, and that the entire Levich affair is merely a peg on which to hang efforts to set the scientific world in the West against the Soviet Union.

This conclusion is inescapable. It is confirmed by the wide circle of persons invited to the conference. Hundreds of people in our country, and maybe even more, have received invitations. Never before, not even when jubilees of the most outstanding Western scientists were marked, did that happen. What is sig-

linked strongly with the work of Levich.

Your paragraph 4 questions the sources of information, and the motives, of the organisers and sponsors of the conference. Our information sources are too numerous to list comprehensively; so examples must suffice. The way in which its Soviet distributors excised Levich's name from an article by Professor Parbury Schmidt (see appendix) is documentary proof of a kind. We have seen the letter from Academician Frumkin relieving Professor Levich from his position, solely because he had exerted his legal right to apply for an exit visa. We have corresponded and held discussions with numerous Soviet scientists, officials and ordinary citizens. . . .

When, we ask you, was Professor Levich last permitted to conduct a seminar in his Institute? When will that permission next be given?

Concerning motives, it is necessary to be blunt: your "inescapable conclusion" is ludicrous; "to set the scientific world in the West against the Soviet Union" is as remote from our intentions as it is beyond our powers. We earnestly ask you to give weight to an alternative possibility, namely that the motives of the sponsors and organisers are commensurate, in probity and good sense, with their scientific attainments and with the

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USSR version on the right

nificant is that many of the recipients of invitations never had anything to do with the theme of the conference, let alone the field of research in which Professor Levich works or science in general. So what does all this mean?

. . . We feel that the whole affair of marking Professor Levich's birthday can do nothing but harm to international cooperation. If the organisers of the conference sincerely desire to strengthen international cooperation, we cannot but ask—with whom? With those who, like Professor Levich, believe for one reason or another that they have been unfairly treated and have become openly hostile to our country, or with the vast majority of scientists who really represent Soviet science and are deeply loyal to our country?

We firmly believe that in deciding to convene this conference its organisers were concerned not with promoting international cooperation between scientists, but with using Professor Levich's 60th birthday to set public opinion against our country. . . .

YA. KOLOTYRKIN, K. GORBUNOVA, B. KABANOV, V. LOSEV, N. D. TOMASHOV, N. SHUMILOVA, V. DMITRENKO, N. LIDORENKO, S. KARPACHOV, J. MATULIS, D. V. SOKOLSKY, J. STRADINS, Y. DELIMARSKY.

dignity of their offices. Please consider well their names; then ask yourselves again: can your inescapable conclusion be correct? We would assure you that if any scientist of the standing of Levich, in any other country of the world, were treated in the same way, our involvement would be exactly the same.

In paragraph 5, you express uncertainty as to whether the effect of the conference on international cooperation in science will be good or bad; and you ask with whom cooperation is intended by us. The answer to the second question is: we hope to cooperate with *you*, and with your colleagues in the USSR; that is why we sent you invitations, and that is why the conference publicity has been kept entirely free from words which might embarrass you.

As to the effect of the conference on relations between Western and Soviet scientists, this depends greatly upon you. We have made plain our equal concern for science and for freedom of movement in all countries. We have disclaimed all provocative intent; and, incidentally, we have provided your government, if it wished to take it, with an opportunity gracefully to extricate itself from an awkward situation. . . .

Yours sincerely,
DEREK BARTON, BRIAN SPALDING

EEC

Research: no stop ahead?

IF THE words of the European Commission are anything to go by, the EEC's involvement in science and technology needs to intensify. Whether or not the Nine member countries of the Community automatically allow such a development is increasingly open to doubt, but the Commission's ideas and hopes for the next few years are now contained in a document which it approved at the end of last month and sent to the Council of Ministers and European Parliament for their consideration.

The document, *The common policy in the field of science and technology*, is in three parts. The core, appropriately enough, is the middle part, and this details the guidelines which the Commission has adopted for the next phase—that is, 1977–80—of Community science and technology policy. With ten chapters and two annexes, it is a detailed if wordy conspectus directed not only at the Council but also “to all those affected by the European research policy”. According to brief final remarks, key areas of Community concern—“such as economic competitiveness, secure energy and raw material supplies and the preservation of a humane social and physical environment”—are becoming “more and more dependent” on a common science and technology policy.

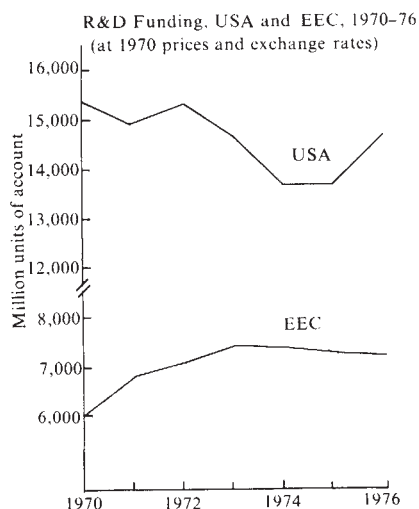
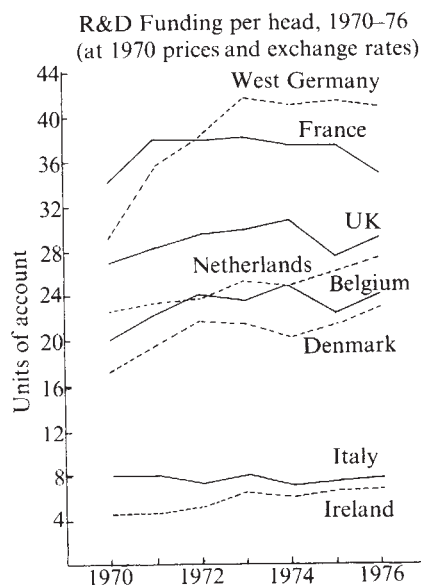
The earlier part of the document contains three draft texts which the Commission proposes that the Council should adopt. One is a resolution on the guidelines that make up the middle part. This says that coordination of R&D shall be

“gradually developed and intensified”; that special attention should be paid to the European Science Foundation's activities; that proposals be made for the better use of R&D results; and that a full review of EEC science activity be undertaken by the end of 1979.

The second text provides for a decision on promotion of industrial R&D projects. This would be done through financial assistance to small- and medium-sized firms cooperating in the sphere of innovation. The third text, which reflects the Commission's feelings that research in forecasting and assessment is essential, provides for a decision on a five-year research programme costing 4.4 million units of account and employing 10 staff. The aim of the programme would be “to contribute to the definition of long term R&D objectives and priorities”.

The final part of the document, from which the accompanying graphs are taken, analyses public financing of R&D and has 15 graphs and four tables that summarise the main trends in the Community over the past six years. They belie the promise of the guidelines. According to the document, there is a general stagnation in R&D funding in all Community countries, an increasing gap between the Nine and the USA, and a gap between collective Community funding of R&D and funding within the individual member countries themselves. Some 64% of Community R&D funds is concentrated on energy; health takes 15–16%, and industry 12–14%.

Chris Sherwell



JET

Is it enough?

A new development came last week in the saga over JET. Chris Sherwell reports

IN WHAT in the absence of confirmation otherwise looks suspiciously like an embarrassing climbdown, Britain last week withdrew one of its last remaining sanctions over the controversial decision on where to site the European Community's Joint European Torus (JET) fusion project. The Council of Foreign Ministers is due to make a final choice between Culham in Britain and Garching in Germany—unless it can come up with a third site as a compromise—at a make-or-break meeting later this month.

Britain has hitherto kept a reserve on the expenditure of about £145 million for the 1977–80 joint research programme of the Community, even though the research programme has itself long been agreed, as a bargaining chip over JET. The official reason has been that the research programme contains a good deal of fusion work, and Britain did not want to see decisions taken “piecemeal”. But an announcement last week from Brussels, which was welcomed by the European Commissioner concerned, Dr Guido Brunner, disclosed that Britain had withdrawn her reserve “in the interests of the Community”, but apparently in recognition that it had become more of a hindrance than an aid to progress on JET.

Whether the move will help Culham get JET is another question. Brunner spoke of “improving the climate of decision-making”, but Britain's handling of matters like these in the past does not exactly augur well. At the energy ministers' meeting in March, for example, a concession over a scheme involving Euratom loans turned out to be rather costly when there was little of the expected progress on another important matter, the minimum safeguard price for oil. On that occasion too Britain's obstruction had by then blackened its reputation. The same could happen again over JET.

Moreover, the new concession may not be enough. In the view of some, a satisfactory decision on JET was possible when the research ministers met in March. The argument is that the political price Britain has to pay to win JET increased at that meeting when, as presidential chairman, she mishandled things and failed to call for a vote—a vote which, it is widely recognised, would then have gone in Garching's favour. □

BRITAIN

● Public fears in the developed world about nuclear power as no less real for the fact that the hazards of over-eating, alcohol, cigarettes and automobiles may be greater. Among those fears the horror of radiation looms large, and if the risk posed by nuclear war is discounted, a nuclear reactor accident must be a favourite worry. The UK National Radiological Protection Board (NRPB) has reviewed the data describing the effects of radiation exposure on a population precisely in order to assess the biological consequences of the accidental release of radioactivity from a reactor. It published the findings last week*.

Information on the effects of radiation in man was derived from various studies—of individuals exposed to the atom bombs dropped on Hiroshima and Nagasaki, of Marshall Islanders contaminated with fall-out, of accident cases in the nuclear industry, and of patients treated by radiotherapy. This was supplemented with data obtained from experiments on animals. Exposure to radiation was presumed to occur in various ways: acute external exposure of the body, whether from the passing cloud or from contaminated ground; inhalation of activity from the cloud and ground and from contaminated food and water; and relocation of incorporated radionuclides within the body.

Depending on the radiation dose, an exposed population is expected to suffer both early ("acute") and late physical damage. Acute effects are minimal below a certain threshold dose. Above that dose, cell membrane damage allows fluids and electrolytes to leak out of the vascular system, and stem cells lose their reproductive capacity, meaning that normal functions can be impaired. The main late effect, cancer, has no threshold; damage to blood vessel linings, hyperthyroidism, cataracts and sterility may also occur.

The report suggests a threshold of about 200 rads (low LET), below which no deaths would occur, for irradiation of the bone marrow. For the lower large intestine a threshold of about 2,000 rads (low LET) is accepted, where the dose is received in the first seven days after exposure and the probability of death covers a one-year period. Pulmonary morbidity is not expected below a threshold dose

over one year of 2,500 rads (low LET).

The report also details estimates concerning late effects. For death from leukaemia it gives a risk coefficient, in cancer deaths per 10^6 man rads (low LET), of 20. Other figures are: cancer of the lung, 20; bone, 10; liver, 10; gastrointestinal tract, 20; breast, 20; thyroid, 5; and all other cancers, 20. The total risk coefficient is 125. The estimates, the report says, are "considered adequate". As for hereditary disease, the report predicts



a total of 57 serious cases per 10^6 man rads (low LET), of which 15 and 9 would appear in the first and second generations respectively.

● Britain and the USA are to collaborate on coal research. The chairman of the UK's National Coal Board (NCB) and the USA's General Electric Company agreed last week to exchange technical information on ways of using coal more efficiently, particularly as an energy source. It is hoped that exchanges on coal gasification, liquefaction, fluidised combustion and the production of metallurgical fuel will eventually lead to jointly run research projects.

British technology is already being used in one US coal gasification project. Work on a gasification process, done at the NCB Utilisation Research Laboratories in Surrey in 1972, is to be incorporated into a recently-approved \$24-million design project which should eventually lead to a \$334-million plant for Illinois to extract gas and low sulphur oil from coal. The Canadians too are using British expertise in coal. The British partnership of the NCB and Woodall-Duckham Ltd is under contract from British Columbia Hydro and Power

Company in Vancouver, Canada, to study the feasibility of designing a demonstration plant for cleanly and efficiently producing electricity and gas from Canadian coal.

Coal, of course, is the less controversial key to Britain's plans to bridge the so-called 'energy gap' in the 1990s (the more controversial one is nuclear power). But last week the chairman of British Gas, Sir Denis Rooke, injected his own element of controversy by dismissing suggestions that there will even be an energy gap at that time. The technologies were being developed, he said, to make substitute natural gas from coal or oil. The energy minister with responsibilities for gas said Britain was already looking to the time when it would have to rely on SNG.

● It is a commonly expressed view that Britain has not been making the most of its professional engineers over the past few years, and that little has been achieved in improving the engineer's lot, especially in manufacturing industry. Now the government has announced a Committee of Inquiry into the engineering profession. Sir Monty Finniston, formerly at the British Steel Corporation, will chair it.

The committee will look at how British industry could better use engineers of varying educational achievement. It will also examine the part engineering institutions could play in relation to the education and qualification of engineers at professional and technician level; the possibility of statutory registration and licencing of engineers in the UK; and the engineering profession in other countries, especially in the EEC. The committee's fifteen or so members have yet to be chosen, but they are expected to convene by the autumn.

Critics have said the inquiry might delay urgently needed changes. But the Secretary of State for Industry, Mr Varley, hopes that the inquiry will not be "unduly prolonged" (it is expected to last 18 months). And if the committee feels that some of its findings deserve urgent attention, it will probably be able to produce interim reports. One question is whether the committee will recommend the establishment of a body to control academic standards and entry to the profession. Professional engineers would then be organised in a similar way to the medical profession, and the new body would be analogous to the General Medical Council.

* H. Smith and J. W. Stather, *Human Exposure to Radiation following the Release of Radioactivity from a Reactor Accident: a Quantitative Assessment of the Biological Consequences* (NRPB-R52, HMSO, 50p).

FRANCE

Mixed fortunes

French nuclear fortunes followed a mixed path last week. A correspondent reports

THE French Cabinet was apparently expected to discuss nuclear issues last week, but reportedly failed to do so. The minor controversy caused by a leak of uranium hexafluoride gas at a plant in southern France couldn't have helped. But there was some glory to bask in too—the signing of a cooperation agreement with West Germany covering fast breeder reactors.

The agreement is not unprecedented, being in line with an arrangement reached in Nice in February last year which involved the two countries pooling risks over both breeders and high temperature reactors. Given the new US policy, however, its timing may be awkward.

A key feature of the latest agreement is the exchange of information on fast breeder R&D over a 20-year period. This involves the French

Atomic Energy Commission (CEA) on the one hand and Interatom, a subsidiary of the German company Siemens, and the Nuclear Research Centre of Karlsruhe on the other. Reports suggest that the French will maintain their fast breeder research effort at existing levels, at about FF400–500 million annually—the Germans pitch in a similar sort of amount—but that both sides are hoping for more efficient use of the funds.

Commercialisation of the fast breeder becomes the object of a new French-dominated company established under the agreement known as Serena. This allows the participation of Belgian, Dutch and Italian interests along with the principal partners from France and Germany.

Paralleling this development is one concerning French uranium supplies. In a deal with a South African corporation, the CEA is reportedly to receive 900 tonnes of uranium oxide over 10 years in return for funding the capital investment programme of the mine which will produce it. □

BANGLADESH

Energy projection

The Bangladesh government recently received a report on its energy prospects. A report from M. Kabir

OVERALL demand for energy is increasing with population growth and with greater industrialisation, and the government of Bangladesh has felt the need for a major study to guide long term policy and advise on specific projects that should be implemented over the next 5 to 10 years. Responding to a government request, the United Nations Development Programme (UNDP) agreed to support and finance through the Asian Development Bank a study involving Canadian, American and Italian firms. This began in mid-1973.

An 8-volume draft of the final report was submitted recently. It considers the energy sector as part of the larger economic system of Bangladesh, attempting to identify interdependence both within the energy sector and between this and other sectors of the economy.

In Bangladesh, about 90% of the people living in the villages consume about 80% of the total energy. But only a small part of total energy requirement for a massive population of 75 million is supplied in commercial

form as electricity, gas or petroleum products. Most derives from the burning of traditional fuels, for which estimation of the energy equivalent is difficult given the lack of statistics.

The existing electric power system consists of two separate zones, the East Zone and the West Zone, divided by the river Jamuna. The most recent year for which data were available for the study was the financial year 1973–74, and in this period generation for public supply amounted to some 1,260 GWh—980 GWh in the East Zone and 280 GWh in the West. In addition there are facilities directly owned by users, known as 'captive power', generation of which in 1973–74 was about 460 GWh, the bulk of it in the East Zone.

In assessing demand a major effort was made in the study to project the likely growth of agriculture. That meant considering the speed with which farmers will adopt new technologies, for example, but also examining the institutional framework within which development occurs. An analysis of demand based on various 'scenarios' in a macro-economic model yielded various projections anticipating population, agricultural production and production in other sectors. The 'mid-demand' scenario, which was adopted as an ap-

CHINA

Conference plan

A NATIONAL CONFERENCE on science and technology is being organised in the People's Republic of China, according to Professor Ren Ci-gong of the Applied Physics Laboratory at Johns Hopkins University. He left China on 30 June after an extended 2½-month tour. Interviewed by reporters in Hong Kong, he said its scale would "greatly exceed anything that we can imagine". During his tour he had come to know of many regional conferences, attended by specialists as well as representatives of the broad mass, and these served as preparations for the planned event.

The news accompanies intense activities in China within the past seven months both in matters of science policy and others involving modernisation of agriculture, industry, communication and defence. On the international level, a Chinese delegation which included such leading scientists as Chien San-chiang of the Peking Institute of Atomic Energy and T. C. Tung of the Institute of Zoology has just returned from a visit to Australia. And last month a team of American scientists headed by Philip Handler, President of the US National Academy of Sciences, went to China.

One of the aims of these exchange visits is no doubt to lay the path for further increases of mutual understanding and cooperation in the field of the natural sciences between the Chinese and other peoples. A long article appeared in *The People's Daily* (Peking) on 30 June signed by the 'Theoretical Group of the Academia Sinica'. In it the validity of a previously published document, "Summary of Reports from the Academia Sinica", was re-affirmed. This was frequently referred to in the campaign to criticise Teng Hsiao-ping, with whose explicit approval it was originally released for internal circulation among state departments. Its content is now widely known and may prove to be a good indication of China's science policy now that the so-called Gang of Four and their followers have been removed from the leadership.

T. B. Tang

appropriate basis for energy planning, projects 3,971 GWh for the East Zone and 2,292 GWh for the West. The equivalent figures on the 'high-demand' scenario were 5,004 GWh and 2,608 GWh. □

IN BRIEF

Azbel in Israel

Mark Azbel, who for the last 2½ years has been running the Moscow 'Sunday seminar' for refusnik scientists, arrived in Israel last week. He said the recent closure of the seminar was not a condition of his obtaining an exit visa, and that he was confident it would reopen shortly.

German report

Professor Heinz Maier-Leibnitz, president of the Deutsche Forschungsgemeinschaft (DFG) has told of the continuing effect of financial cutbacks on Germany's research effort. Writing in the 1976 annual report, published at

the end of last month, he says the "awareness of being at the mercy of something arbitrary in [research] promotion, wherever it comes from, and not to know any more whether a good plan will even be welcomed, easily leads to discouragement and bitterness".

Of the DM646.1 million the council had at its disposal last year (an increase of DM26.1 million on 1975), DM367.6 million came from the Federal government and DM269.9 million from the Lander.

European scrutiny

Well over a year after welcoming the suggestion that other House of Com-

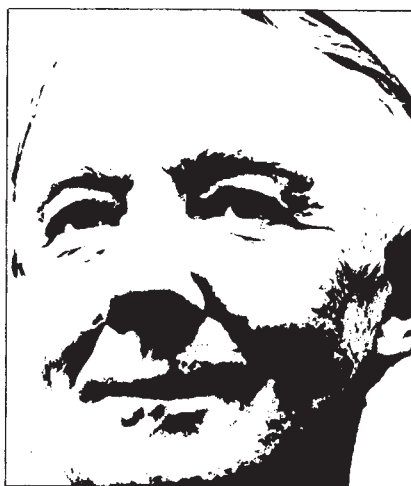
mons Select Committees apart from the existing Committee on European Legislation should consider proposals coming from the European Commission, the Select Committee on Science and Technology has decided to "consider the merits of EEC instruments falling within their order of reference". But the committee's special report announcing the decision, published last week, indicates the sort of fears about divisions of responsibility that may have caused the delay. Such work, the report insists, "cannot supplant the examination of such instruments by parliamentary representatives in the Energy and Research Committee and other appropriate Committees of the European parliament".

Air pollution has both local and international effects. Smoke and dust are usually urban problems, less serious the further one moves from their source. Fluoride, from brick-works and aluminium smelters, seldom causes damage beyond a radius of fifteen kilometres. But sulphur can be transported for hundreds of kilometres, over frontiers and oceans, and may cause international disagreements. It is well known that scientists in Norway and Sweden complain that sulphur emissions from Britain, Germany, Czechoslovakia and Poland have turned their rain and their rivers acid, harming freshwater fisheries, and possibly reducing growth in their coniferous forests. Many people have the impression that the industrial countries have developed a technique for keeping their own air relatively clean and at the same time causing gross pollution to harm the innocent Scandinavians.

It is true that, though there has been little decrease in the total emission of sulphur by industry and from domestic heating in Britain over the last twenty years, levels of sulphur dioxide, SO_2 , in our cities have fallen as the gas has been increasingly discharged from high chimneys. This can be shown by the way the disease 'black spot' has returned to damage roses in urban gardens, where previously the fungicidal properties of the air kept it in check. But though the sulphur is certainly more widely dispersed, the bulk of it remains near to its source. British or German air is much more heavily charged with sulphur than that which crosses the sea to Norway and Sweden. British rain is generally acid. Even in rural Cambridgeshire measurements of pH 4 and lower

are common; some 70 kg of sulphur is deposited on average on every hectare of Britain, and more than 20 kg in the most rural farming areas. The amounts deposited in Scandinavia are much lower. Yet

Acid rain



KENNETH MELLANBY

damage in Britain is rare, and widespread in Norway and Sweden. The situation is clearly complicated.

In Britain most of the sulphur polluting the atmosphere is gaseous SO_2 . Even in the cleanest areas annual average levels of $20 \mu\text{g m}^{-3}$ are recorded. These are generally believed to have no harmful effects. None have been detected with crops or animals, and even foliose lichens, plants known for their extreme sensitivity to sulphur, flourish. In southern Norway, SO_2 levels are seldom as high as $5 \mu\text{g m}^{-3}$, and, as we would expect, lichens grow well. By British standards, the air is remarkably pure. The SO_2 hardly

seems to be the villain.

The main cause of acid rain seems not to be SO_2 but sulphate. In Britain amounts of sulphate are, in comparison to SO_2 , low, and probably of little importance. In Scandinavia most of the airborne sulphur is present as sulphate, much derived from SO_2 produced by European industry and transformed during the period of passage north. This is washed out by the heavy rainfall. As already mentioned, the rain is actually less acid than that falling in Britain, and the total amounts of sulphur are generally small (less than 10 kg per hectare). Why then should there be all this fuss?

There are two main reasons. First, most water in Britain is well buffered, and it and most soils can neutralise most of the (comparatively large) amounts of sulphur pollution. Most Scandinavian freshwater is very 'pure' with little buffering power, so a little additional acid can greatly reduce the pH. Also much rain is stored as snow for up to six months; when it thaws the pollution of half a year can be suddenly discharged.

There is no easy solution. Industrial countries can reduce levels of sulphur emission sufficiently to eliminate damaging pollution within their own borders, but will find it difficult to prevent the small fractions of their emission which escape overseas from damaging the rivers of Scandinavia. It is ironical that this sulphur can also contribute to agricultural productivity. Many Scandinavian soils are sulphur deficient and to optimise crop yields this element must be added as a fertiliser unless adequate amounts are deposited from the atmosphere. What is pollution for the fish may be an essential growth factor for the cereals.

correspondence

Preparing scientific papers

SIR,—A great deal of time is wasted because young scientists submit papers for publication in an unacceptable form. There are many good books on the market on the preparation of scientific papers, but few specific examples as to how an unacceptable manuscript can be transformed into one acceptable by leading scientific journals. By a lucky chance (and completely legally) I was able to obtain a copy of such a manuscript with the referees comments and a model re-written version, from the editorial files of a leading geological journal, and I pass this on in the hope that it will be of value to authors in preparing papers for publication.

Columnar Rock Structures from an Antique Land

Referees' report:
manuscript 19705B/76: P. B. Shelley

Manuscript as submitted:

Ozymandias¹ by P. B. Shelley²
I met a traveller² from an antique land³
Who said: Two⁴ vast⁵ and trunkless legs⁶
of stone⁷
Stand in the desert.⁸ Near them⁹ on the
sand¹⁰
Half sunk, a shatter'd visage lies whose
frown
And wrinkled lip and sneer of cold
command
Tell that its sculptor well those passions
read
Which yet survive stamp'd on these
lifeless things,
The hand that mock'd them and the
heart that fed.¹¹
And on the pedestal¹² these words appear:
"My name is Ozymandias, King of Kings.
"Look on my works, ye mightily, and
despair."¹³
Nothing beside remains¹⁴; boundless¹⁵
and bare
The lone and level sands¹⁶ stretch far
away.

Referees' comments

¹This title is quite inadequate. Includes no keywords. See suggestion below.

²Since this paper appears to be based on field observations by another geologist, we suggest joint authorship would be appropriate.

³Specify.

⁴This is the only quantitative statement!

⁵Not specific enough. The authors should give dimensions in SI units. (Unless "vast" is a class in some sort of grade-scale, in which case, the reference to this scale should be given.)

⁶Have alternative hypotheses been considered? Earth pillars? Basalt columns? Ant hills?

⁷Surely identification of rock type with appropriate analyses could be provided here?

⁸Give co-ordinates.

⁹Specify distance. A photograph (giving scale) would help here.

¹⁰Give granulometric analysis, and preferably some scanning electron microscope photographs of grain-surface textures. These don't actually prove anything, but are decorative and keep SEM operatives in employment.

¹¹This fanciful and speculative section could well be omitted.

¹²This is the first we have heard of a pedestal!

¹³While it may be worth-while to record the defacement of an interesting exposure, it is not necessary to quote the words. (Since they are in English, they are obviously of no archeological interest. Presumably graffiti sprayed on by a tourist).

¹⁴Rather dogmatic. Better: "No other rock exposures were observed".

¹⁵Inappropriate hyperbole. The approximate extent of the desert should be stated, if relevant.

¹⁶Unless this is a windless desert, surely a sandy desert should show dune formation? If actually level, perhaps in fact it is a stony desert?

General remarks Although some interesting observations are recorded, we cannot recommend publication in the present form. For one thing, the paper is far too short. For another, the authors have inexplicably left out any mention of plate tectonics! For the guidance of the authors, we give below a summary of the kind of re-written and expanded paper which might be acceptable. We have had to supply necessary missing data arbitrarily; it is, of course, up to the authors to substitute the correct data.

Suggested re-written manuscript (summary)

Twin limb-like basalt columns ('trunkless legs') near Wadi Al-Fazar, and their relationship to plate tectonics
Ibn Batuta¹ and P. B. Shelley²

In a recent field trip to north Hadhramaut, the first author observed two stone leg-like columns 14.7 m high by 1.8 m in diameter (medium vast, ASTM grade scale for trunkless legs) rising from sandy desert 12.5 km southwest of Wadi Al-Fazar (Grid 474 753). The rock is a tholeiitic basalt (table 1); 45 analyses by neutron activation technique show that it is much the same as any other tholeiitic basalt (table 2). A large boulder 6 m southeast of the columns has been identified as of the 'shattered visage' type according to the classification of Pettijohn (1948, page 72). Granulometric analysis of the surrounding sand shows it to be a multimodal leptokurtic slightly positively skewed fine sand with a slight but persistent smell of camel dung. Four hundred and seventy two scanning electron photomicrographs were taken of sand grains and 40 are reproduced here; it is obvious from a glance that the grains have been derived from pre-cambrian anorthosite and have undergone four major glaciations, two subductions, and a prolonged dry spell. One grain shows

unique lozenge-shaped impact pits and heart-like etching patterns which prove that it spent some time in upstate New York.

There is no particular reason to suppose that the columns do not mark the site of a former hotspot, mantle plume, triple junction, transform fault, or abduction zone (or perhaps all of these).

Keywords: plate tectonics, subduction, obduction, hotspots, mantle plume, triple junction, transform fault, trunkless leg, shattered visage.

¹School of Earth & Planetary Sciences, University of the Fertile Crescent.

²formerly of University College, Oxford.

Yours faithfully,

N. S. HAILE

University of Malaya,
Kuala Lumpur, Malaysia.

The laetrile question

SIR,—When Thomas H. Jukes came to witness our laetrile hearings (19 May, page 201), he missed the main battle and reported only the smokescreen. As an American, a scientist and a non-believer in laetrile, let me give an alternative view.

Support for freedom to use harmless drugs is no more a "crushing defeat to science and rationality" than is freedom to seek medical miracles through an established religious faith. The laetrile controversy brings into play three considerations:

- History is replete with examples where the consensus of established scientists was wrong.

- There is considerable resentment of the wealth that medical doctors extract by virtue of their monopoly control over the keys to life and health; prescriptions, laboratories and the like.

- Many people in the United States strive for freedom from excessive government; we seek a compromise between freedom and control in which individuals can pursue happiness as they see fit.

The laetrile question strikes all three chords. "Shall the government grant to doctors the exclusive right to dispense or withhold a drug which they believe to be harmless?" Would Jukes turn people away from Lourdes? Surely the answer is no, and just as surely the answer is no defeat for science.

Yours faithfully,

THOMAS A. CROFT

Atherton, California, USA

news and views

Adenovirus amazes at Cold Spring Harbor

from J. Sambrook

The XLII Cold Spring Harbor Symposium on Quantitative Biology, entitled Chromatin, was held on 1-8 June, 1977. New insights into the ways in which gene expression is controlled in adenovirus were reported at the symposium and are described below.

If success were exactly proportionate to effort invested, our understanding of the mechanism of gene expression in eukaryotes would be virtually complete. Unfortunately, despite intensive investigations for a number of years, our ignorance remains almost total. In part, this frustrating situation stems from the technical difficulty of analysing the expression of genes which may be represented only once in a huge and complex genome. However, the continuing dearth of useful mutations in control regions and the failure to reconstitute *in vitro* systems in which synthesis of mRNA or its precursors is initiated have also been strong contributing factors.

Despite these difficulties, there is circumstantial evidence of several sorts to indicate that extensive control of gene expression occurs at the level of mRNA synthesis. For example, mRNAs which code for specific differentiated products are detectable only in those cells which are committed to make those products. Thus, globin mRNAs are found only in reticulocytes, ovalbumin mRNAs only in cells of the oviduct and so on (see *Nature, News and Views* 267, 203; 1977). Two competing theories have been used to explain how such gene regulation may occur. The first, based on experience with prokaryotic systems proposes that different regions of the cell's genome are transcribed into mRNA at different frequencies and that mRNA is either the direct product of the transcription event or is derived from precursors which need not be much larger than the mature mRNA

itself. This scheme places control of gene expression firmly at the level of transcription, implies that there are control elements before each gene, and relegates post-transcriptional modification to a subsidiary role. The second hypothesis on the other hand suggests that mRNAs are derived from extremely long precursor molecules (identified with heterogeneous nuclear RNA) by a complex set of post-transcriptional steps involving specific endonucleolytic cleavage and modification of the 3' and 5' ends of the RNA by addition of poly(A) and methylated bases. This pathway, in which less emphasis is placed on transcriptional control, is attractive because it provides a plausible reason for the existence of mRNA.

To test these ideas, several groups of workers have studied gene regulation in animal viruses as a model system which might be expected to reflect the processes that occur in eukaryotic cells. Recent findings from one of these groups indicate that both mechanisms of control may be active.

Most of the data have been obtained with adenovirus 2, a human virus which grows well in cultured cells. Its genome consists of a linear complex of DNA about 35,000 base pairs in length, sufficient to code for 30-40 polypeptides of average size. The expression of these polypeptides is under temporal control: some of them (confusingly called early proteins) are synthesised at all times during lytic infection; others (late proteins) are synthesised only at late times. The early genes are collected into four widely-spaced regions and during the early stage of infection the only virus-specific mRNA present in the cell is complementary to one strand of the DNA sequences of these four regions (for a review see Flint *Cell* 10, 153; 1977).

At the Cold Spring Harbor symposium, J. E. Darnell (Rockefeller University) presented evidence to show that each of these four groups of early genes has at least one promoter sequence. Nuclei were isolated from cells early during lytic infection and

incubated in the presence of radioactive precursors of RNA in conditions which do not allow the initiation of new RNA chains, but merely the extension by a hundred or so nucleotides of those already begun in the intact cell. At the end of the brief incubation period the RNA was extracted and divided into different size classes by sedimentation through sucrose gradients. The shortest RNA molecules to be labelled *in vitro* should be those that had just begun to be synthesised *in vivo*. By hybridising the short RNA chains to different segments of the viral genome, obtained by cleavage of adenovirus 2 DNA with restriction endonucleases it was a straightforward task to identify the sites at which RNA synthesis had begun. Darnell and his coworkers (R. Evans, N. Frazer, S. Goldberg, T. Weber and N. Wilson) found four starting points for early RNA—two on the *r* strand at positions 1.8 and 80 and two on the *l* strand at positions 98 and 72. Because they lie at the beginning of the genes which code for early proteins it seems likely that these four regions serve as classical promoters—sequences which direct RNA polymerase to begin synthesis of an RNA chain. When infection is stringently blocked in the early phase, no viral RNA sequences are detectable in the nucleus other than those copied from the early regions. Thus expression of early viral genes seems quite clearly to be under transcriptional control.

At late times after infection a very different situation obtains. After viral DNA synthesis begins there is a vast increase in the rate of viral RNA synthesis as well as changes in the pattern of transcription. The cytoplasm contains a variety of new RNA species 1,000-4,000 bases long, that together account for virtually all the information of the viral genome. In addition the nucleus contains high molecular weight, virus-specific RNA molecules, some of which seem to be more than 20,000 bases long. More than 80% of both nuclear and cytoplasmic transcripts are complementary to one strand of

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viral DNA—the *r* strand. In two kinds of experiment described in the April issue of *Cell*, Darnell and his coworkers (Weber, Jelinek & Darnell *Cell* **10**, 611; 1977; Goldberg, Weber & Darnell *Cell* **10**, 617; 1977) have shown that the synthesis of the vast majority of the giant nuclear viral RNA molecules is initiated at a specific site on the adenovirus genome. Once again nuclei were isolated, this time from cells in the late stages of infection, and RNA chains were extended by the addition *in vitro* of a few hundred labelled nucleotides. The radioactive label incorporated into the longest RNA molecules was found to hybridise only to the right hand end of the viral genome: the shortest molecules, however, hybridised preferentially to a region mapping between positions 14.5 and 19.0 on the viral genome. Thus late viral RNA complementary to the *r* strand is synthesised from one long transcription unit, with a promoter near position 14.5 and a terminator near the right hand end of the viral DNA. This conclusion is confirmed by experiments in which infected cells were exposed to ultraviolet irradiation—a treatment which causes lesions in DNA through which RNA polymerase cannot pass.

The frequency at which a particular sequence of DNA will be transcribed will therefore depend on whether a lesion is introduced between the sequence in question and its promoter. If the lesions are randomly distributed throughout the population of viral DNA molecules, transcription should decrease exponentially with distance from the promoter. Late adenoviral RNA synthesised immediately after irradiation was found to hybridise most efficiently with sequences from the left hand 30% of the viral genome and progressively less well with sequences to the right. The results indicate that a late promoter is present near the left hand end of the *r* strand of adenoviral DNA.

Taken together, both types of experiment show, first, that late in adenoviral infection, transcriptional control still occurs, and second, that the site at which RNA synthesis begins is quite specific and efficient. By contrast to the early promoters however, the late promoter near 14.5 on the *r* strand is highly polar and controls the expression of all genes to its right. The primary product of transcription is a long RNA molecule complementary to DNA sequences between 14.5 and 100, which is cleaved endonucleolytically to yield mature mRNA. The shift from the early to the late phase of adenovirus transcription must therefore involve alterations not only in the genes that are expressed but also in the method by which their expression is controlled.

Mass of Saturn's rings

from David W. Hughes

MASS is one of the more awkward quantities to measure in an astronomical context. Usually it is derived from a careful analysis of the perturbing effect the gravitational field of one body has on the orbit of another or, less satisfactorily, by simply multiplying the volume of the object by an estimate of its density. Inevitably the problems and uncertainties increase as the body becomes smaller or more tenuous, so W. I. McLaughlin and T. D. Talbot from the Jet Propulsion Laboratory, California are to be congratulated on obtaining the reasonably precise value of $(3.5 \pm 1.4) \times 10^{24}$ g for the mass of the rings of Saturn. Their calculations have been published in a recent edition of the *Monthly Notices of the Royal Astronomical Society* (**179**, 619; 1977).

The gravitational potential around Saturn is a function of the planet's mass, size and principal moments of inertia and also of the mass, size and orbits of the rings and the satellites. A knowledge of the rate of precession of the satellite orbits enables this potential to be estimated with considerable accuracy. Reliable observations of the six inner satellites, Mimas, Enceladus, Tethys, Dione, Rhea and Titan going back as far as 1888 have been analysed by Kozai (*Annls Tokyo astr. Obs. Ser.* **2**, 5, 72; 1957) and Garcia (*Astr. J.* **77**, 684; 1972) to give the precession rates of the line joining the ascending and descending nodes and also the line joining the apsides.

Using these and also values for the masses and semi-major axes of the satellites, McLaughlin and Talbot have solved the gravitational potential energy function to give values for Saturn's J_2 and J_4 (coefficients in the power expansion of the potential energy function, J_2 being directly related to the moments of inertia about the spin axis and an axis in the equatorial plane) and for the mass of the rings and of the satellite, Rhea. The first solution they presented however did not use a model for the mass distribution inside Saturn and

had only a limited usefulness as far as calculating J_4 and the ring mass goes, given the latter imprecisely as $(5 \pm 33) \times 10^{24}$ g.

A much more meaningful solution was obtained by incorporating the results of Hubbard, Slattery and DeVito's high precision modelling of the interior of Saturn (see *Astrophys. J.* **199**, 504; 1975) which led to a direct functional relationship between J_2 and J_4 . The inclusion of this gives a ring mass of $(3.5 \pm 1.4) \times 10^{24}$ g, a mass for Rhea of $(2.7 \pm 0.5) \times 10^{24}$ g and values of $(1.664 \pm 0.001) \times 10^{-2}$ and $-(8.7 \pm 0.1) \times 10^{-4}$ for J_2 and J_4 . The mass obtained for the rings is very close to the value obtained by Franklin, Colombo and Cook (*Icarus* **15**, 80; 1971) who used a completely different calculation based on the observations of the location of the Cassini division which occurs between the A and B ring. They found a 1,400 km discrepancy between the observed position and that predicted by assuming that the division was due to particles orbiting at that distance being in resonance with the orbit of Mimas, and thus being quickly removed by gravitational perturbation. They concluded that the shift was caused by the self gravitation of ring B thus leading to an augmentation of its outer boarder. To cause the observed shift they calculated that the rings had to have a mass not less than 3.4×10^{24} g a value which agrees surprisingly well with the McLaughlin and Talbot values of $(3.5 \pm 1.4) \times 10^{24}$ g.

So in a mass league of planetary companions the rings of Saturn come in ninth, behind Ganymede, Triton, Titan, Callisto, Moon, Io, Europa and Titania. Also if all the mass in the rings were put together into one satellite with a density say like that of Dione and Rhea ($\sim 2.4 \text{ g cm}^{-3}$) this satellite would have a radius of around 700 km.

David W. Hughes is a lecturer in the Department of Physics at the University of Sheffield.

Following the leader

How mRNAs are cleaved from their nuclear precursors is totally unknown, but bizarre processes must be involved. The audience at the symposium was amazed, fascinated and not a little bewildered to learn that late adenovirus mRNAs are mosaic molecules consisting of sequences complementary to several non-contiguous segments of the viral genome. In every case so far examined, late adenovirus mRNAs contain at their 5' ends about 150 nucleotides which are transcribed not

from DNA sequences immediately adjacent to the structural gene, but from sequences which map at distant locations in the viral genome.

P. A. Sharp (Massachusetts Institute of Technology) and his colleagues, S. N. Berget, A. J. Berk and T. Harrison, described experiments in which purified mRNA coding for the major coat protein, hexon, was annealed to a restriction fragment (*HindIII* A) of adenoviral DNA which spans the hexon gene. When the resulting hybrids were examined by electron microscopy, only

15% were found to consist of perfect duplex structures. Protruding from the vast majority was a tail of RNA which was shown by its location and length to consist of about 150 nucleotides at the 5' (or leading) end of the mRNA. All attempts to remove the leader sequence by incubating hexon mRNA in conditions which destroy RNA-RNA hybrids were unsuccessful, and Sharp and his colleagues were forced to conclude that the leader sequences are not complementary to DNA sequences which lie immediately upstream from the hexon structural gene. The full complexity of the situation was not apparent until hexon mRNA was hybridised to the isolated *r* strand of *Eco*RI fragment A DNA (see Fig. 1). Consistently observed were hybrids whose structure could be interpreted only as shown in Fig. 2a.

These results mean that mature hexon mRNA consists of four separate blocks of sequences. Measurement of the lengths of single-stranded DNA and DNA-RNA hybrids show that closest to the 5' end of hexon mRNA is a small number of nucleotides complementary to position 16.8 on the *r* strand of the viral DNA: next come approximately 80 nucleotides coded by position 19.8; then 110 nucleotides from position 26.9 and finally the coding sequences of the hexon gene itself which maps between positions 51.7 and 61.2. The structure of the hexon mRNA is shown in Fig. 2b.

An identical conclusion was reached independently and simultaneously by a number of groups working at Cold Spring Harbor itself, using several different late adenoviral messengers and a variety of different assay systems. Their results may be summarised as follows:

- many if not all adenoviral late mRNAs contain an identical capped oligonucleotide (7Me Gppp ACU [C₁U₃] Grp) at their 5' end (R. E. Gelinas, R. J. Roberts)
- these capped structures are not complementary to viral DNA sequences near the major late structural genes but hybridise with DNA sequences which map between positions 14.7 and 21 (D. S. Klessig)
- many late adenoviral mRNAs which function in a cell-free system, hybridise not only to the appropriate structural gene, but also to DNA sequences which map between 16 and 32 (J. Lewis, A. Dunn, J. Hassell)
- mRNAs for fibre, 100 K, hexon and penton each contain sequences at their 5' ends which can be seen by electron microscopy to hybridise to positions 16.7, 19.7 and 26.7 on the *r* strand of the adenoviral genome (L. T. Chow, Gelinas, T. R. Broker & Roberts).

These unexpected findings raise a number of interesting questions. How

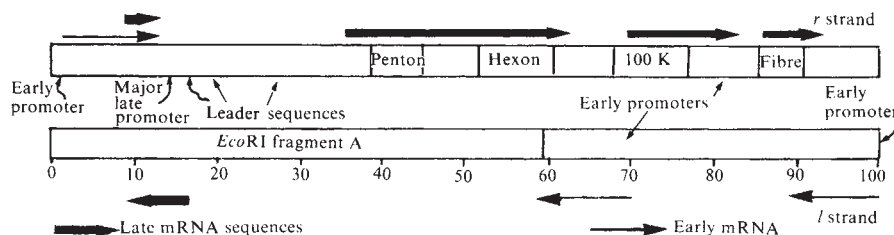


Fig. 1 Transcription map of adenovirus 2 (see Flint *Cell* 10, 153; 1977).

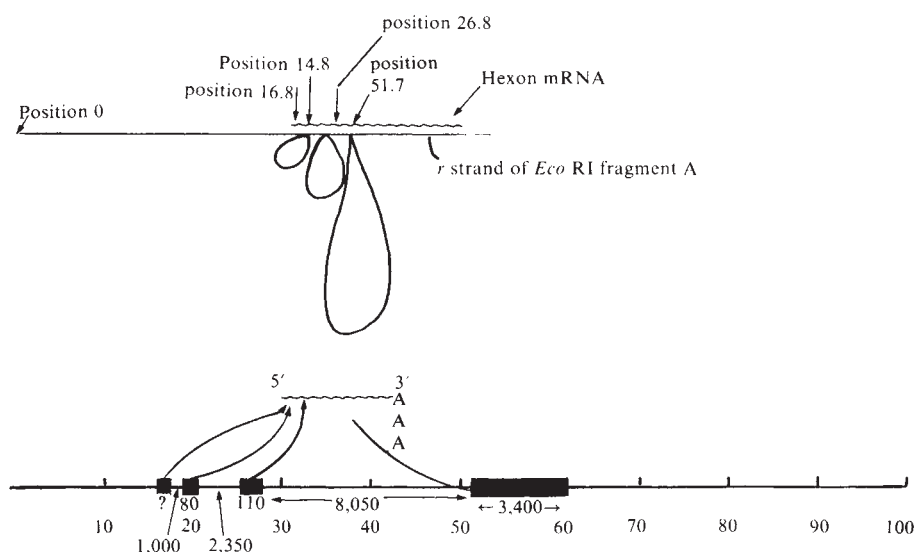


Fig. 2 a, Pattern of hybridisation between hexon mRNA and the *r* strand of *Eco*RI fragment A of adenovirus 2 DNA. b, Regions of adenovirus genome which contribute to hexon mRNA. Figures other than adenovirus DNA markers represent distances in nucleotide base pairs.

are the mosaic molecules synthesised? Why are the three leader sequences separated from each other and from the structural genes? Do the leader sequences serve any function? How general is the phenomenon?

At the symposium four possible ways were discussed by which mosaic late adenoviral mRNA might be synthesised: first, the polymerase responsible for late adenoviral transcription might jump from one segment of the viral genome to another in an ordered fashion. Second, there might be new DNA templates generated, perhaps by recombination, that would contain the appropriate arrangements of different segments of the viral genome. Third, each of the segments may be transcribed independently and the mature mRNAs may be synthesised by ligation of components. Fourth, all late mRNAs that contain an identical leader sequence may be derived by cleavage from the same nuclear

precursor.

The first three hypotheses are inconsistent with the data of Darnell's group discussed above. The fourth hypothesis, however, is identical to the conclusions drawn by Darnell and his coworkers. All the mRNAs that have been shown so far to share the same leader sequence also belong to the same transcription unit and fall under the control of the promoter located on the *r* strand of viral DNA near position 14.5. By contrast a small late mRNA coded by sequences mapping between positions 9.7 and 11.1 on adenovirus 2 DNA and whose synthesis cannot be controlled by the promoter at position 14.5, contains a leader sequence which hybridises to DNA between positions 5.0 and 6.4 (Chow, Broker, Roberts & Westphal).

The best mechanism to explain these findings seems to be that espoused by Klessig. He believes that there may occur in the primary transcript, base-

pairing between the leader sequences and the coding sequences so that mature mRNA would be derived by recombination-like events. Each precursor would then generate one mature mRNA. Perhaps separation of the several leader sequences and those of the structural genes allows several different modes of base pairing whose relative frequencies may determine the rates at which different mRNAs are produced.

The major problem is to find out

how widespread the phenomenon is. Already there is evidence presented at Cold Spring Harbor by S. Weissman, Y. Aloni, G. Khoury and Ming Ta-Hsu that a similar situation may occur during synthesis of late SV40 mRNAs. But the key question is whether analogous events happen in uninfected cells. Perhaps it is not too optimistic to hope that what has been so clearly shown for adenovirus 2 may also help to explain how host cells regulate their own genes. □

Genes, channels and membrane currents in *Paramecium*

from Roger Eckert

THE ciliated slipper animalcule first observed by Anton van Leeuwenhoek in 1674, and familiar to generations of introductory biology classes and students of cytoplasmic inheritance, has lately proved increasingly useful for novel interdisciplinary approaches to problems of broad neurobiological interest. This is not a completely new departure, however, for the very first intracellular electrical measurements from animal cells were made on *Paramecium* over 43 years ago by T. Kamada (*J. exp. Biol.* **11**, 94; 1934), a pioneer in the use of saline-filled capillary microelectrodes. More recently the surface membrane of this unicellular organism was found to exhibit properties of electrical excitability (see review in *Calcium in Biological Systems* ed. Duncan, C. J. page 233 Cambridge University Press, 1976), similar in essence to those shown by metazoan nerve and muscle preparations. Most prominent is a graded depolarisation produced by an inward regenerative current in response to depolarising stimuli. The entry of Ca ions carrying this current produces a transient rise in intracellular [Ca] that mediates a reorganisation of axonemal activity seen as reversed ciliary beating with backward swimming. The stimulus-response coupling sequence is summarised in Fig. 1.

As in the classical excitable membranes of nerve and muscle, depolarisation leads to an inward current (carried in *Paramecium* by Ca) followed by a more slowly developing outward K current. The electrically excitable membrane channels that carry

the Ca current seem to reside almost exclusively in the portions of the surface membrane covering the cilia, for removal of the cilia eliminates the inward Ca current, which is restored gradually as this portion of the membrane regenerates with ciliary regrowth over a period of several hours (Dunlap & Eckert *J. Cell Biol.* **70**, 245a; 1977). Interest in Ca channels is heightened by reports of their occurrence and functioning in sensory, nervous, secretory, embryonic and motor tissues, and by growing evidence that Ca entering cells often acts as a second messenger in modulating certain metabolic activities as well as membrane conductances (Baker in *Calcium Movement in Excitable Cells*, Pergamon Press, Oxford, page 7; 1975; also various articles in *Calcium in Biological Systems* *op. cit.* Cambridge University Press, 1976).

Paramecium offers a unique combination of features useful for new approaches to the study of the Ca system and other components of excitable membranes. As a microorganism it can be mass cultured, cloned and manipulated and analysed genetically. It also possesses the fundamental electrical properties of an excitable cell. Because Ca channels are localised in the membrane covering the cilia a greatly enriched channel fraction can be produced by collecting the cilia which have a high surface area/volume ratio. Regrowth of the cilia provides a model system for studies of channel regeneration and insertion into the membrane. In addition, the coupling between membrane function and ciliary activity facilitates identification and isolation of membrane mutants by their sensory and locomotor behavioural phenotypes. The sensitivity of the ciliary apparatus to intraciliary [Ca] also provides an independent assay of Ca entry through

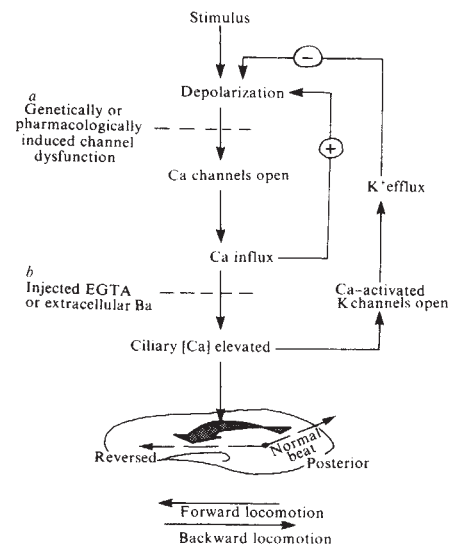


Fig. 1 Relations between channels, currents, and locomotor activity in *Paramecium*. The stimulus-response coupling sequence, which proceeds from top to bottom, can be interrupted (dashed lines) in various ways; for example, by blocking the Ca channels (a) or by interference with intraciliary accumulation of free Ca²⁺ (b). Barium ions, like Ca²⁺, promote ciliary reversal, so they do not prevent reversed swimming, although their substitution for intraciliary Ca does interfere with activation of the Ca-dependent K channels, causing Ba to promote long-lasting all-or-none action potentials with accompanying ciliary reversal in place of the brief, graded Ca response normally produced by the membrane. EGTA injection, by limiting Ca concentration buildup, interferes with both ciliary reversal and activation of the Ca-dependent K channels. This can produce prolonged all-or-none action potentials without ciliary reversal. A report in this issue (Oertel *et al. op. cit.*) demonstrates the utility of a membrane mutant in which a genetically determined Ca channel dysfunction (a) is used to analyse the normal behaviour of the membrane.

the channels of the ciliary membrane (see below).

This issue of *Nature* contains an excellent example of the clever genetically-assisted analyses of membrane function that are being carried out on *Paramecium tetraurelia* by Ching Kung and his associates at the University of Wisconsin (Oertel *et al.* page 120). Membrane currents in response to step depolarisations of the wild type were compared with those of *pawn*, a class of mutant in which the Ca channels had been shown previously to be functionally defective. Predictably, the *pawn* membrane produced only the delayed outward K current, whereas the wild type produced, in addition, the previously reported early inward current carried through Ca channels. Averaged *pawn* current trajectories

were graphically subtracted from averaged wild-type trajectories in order to produce a difference trajectory representing the currents that are missing in the mutant. This approach is similar in concept to isolation of partial currents with the aid of ionic or pharmacological blocking agents (Fig. 1, point *a*), which, regrettably, have not worked well to date on *Paramecium*. From these analytical procedures, Oertel *et al.* concluded that the Ca current completely and rapidly inactivates within milliseconds rather than persisting under prolonged depolarisation, and that a prolonged Ca-activated K current, which might conceal the Ca current, is also absent in the wild-type membrane.

This work is noteworthy not only because it introduces a unique and interesting genetically-assisted approach to membrane current isolation, but also because it has led to important conclusions that seem to disagree with conclusions arrived at through less elegant but perhaps equally reliable means (Fig. 1, point *b*). Thus in simple membrane voltage recordings, injection into *Paramecium* of the Ca-chelator EGTA, or extracellular addition of barium ions converts the normally brief, graded Ca response to an all-or-none action potential exhibiting prolonged plateaux due to delayed repolarisation. In molluscan neurones such behavior is ascribed to diminished activation of a Ca-activated outward K current (Meech *Comp. Biochem. physiol.* **48A**, 387; **48A**, 397; 1974), and concomitant exposure of a persistent inward Ca current that normally is short-circuited by and can be obscured by the Ca-activated K current (Eckert & Lux *J. Physiol.* **254**, 129; 1976).

The cilia, which are sensitive to the intraciliary Ca concentration, beat steadily in reverse throughout voltage clamp depolarisations for periods lasting from tens to tens of thousands of milliseconds, but revert quickly to non-reversed beating characteristic of the low resting intraciliary [Ca] on repolarisation (Machemer & Eckert *J. comp. Physiol.* **104**, 247; 1975). This precise temporal coupling of Ca-dependent, reversed beating to membrane depolarisation in *Paramecium* suggests that a Ca current continues to flow during depolarisation, maintaining an elevated intraciliary Ca concentration. Such a Ca current can easily be overlooked in current measurements if short circuited by a Ca-activated K current (see for example Clusin & Bennett, *J. gen. Physiol.* **69**, 145; 1977). Rapid shut down of Ca channels (Eckert *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 1748; 1977) could make it difficult to measure the end-of-pulse tails of a persistent current. Other factors that

might contribute to apparent or real Ca inactivation are: (1) the negative-going shift in the Ca equilibrium potential with attending drop in EMF driving the Ca current that occurs as the Ca activity within the restricted space of the cilia rises during Ca entry; and (2) a possible depression of the conductance of the Ca channel due to accumulation of Ca within the channel or at sites on the cytoplasmic side of the membrane. Such self-limiting behaviour may be absent with injected EGTA or with extracellular barium.

These and similar problems in sorting out partial currents are not limited to *Paramecium*, of course, so it will be of general interest to see how they are resolved. In any event, the report by Oertel *et al.* reminds us again that genetically assisted approaches to neurobiology, first applied to metazoan material by Benzer, Pak, Nirenberg, Nelson, Brenner and their associates, are not restricted to multicellular animals that possess a nervous system. It seems likely, in fact, that combined genetic, electrophysiological and biochemical approaches facilitated by the unique and fortuitous combination of properties exhibited by *Paramecium* and its relatives will contribute significantly to our understanding of excitable membranes. □

NMR of macromolecules

from Paul Calvert

The Euchem Conference on The Study of Macromolecules by NMR was held at Grasmere on 17-21 May, 1977.

NMR is no longer of wide interest as an effect in its own right but only in so far as it can yield useful chemical information. While the Euchem Conference provided no fireworks it did give a good general view of what NMR can now say about macromolecules and what can be expected in the near future. The usefulness of NMR to the study of macromolecules has increased in recent years with the development of commercial pulsed Fourier transform instruments and high field superconducting magnet spectrometers. High field (> 6.3 T) spectrometers are required to produce well-resolved spectra of high molecular weight polymers because their relatively slow motion in solution allows proton-

proton interactions to broaden the lines. (1 Tesla = 10^4 Gauss. The Larmor frequency of protons is 100 MHz at 2.4 T.)

Synthetic polymers

The meeting was divided into synthetic polymers, molecular motion, and biological polymers, but in essence the subject has two branches: first, the effects of chemical modification on peaks obtained from resolved spectra, and second, determination of the side group and main chain motions from the peak relaxation times. H. J. Cantow (University of Freiburg) introduced synthetic polymer structure studies, pointing out that there were now more than 100 papers describing primary structure determination in polymers by ^{13}C NMR. ^{13}C chemical shifts of aliphatic hydrocarbons cover a wide range and are additive, which has made this the standard technique for structural characterisation of vinyl polymers and copolymers; especially in industrial laboratories for the study of the effects of polymerisation conditions on structure. For instance, in ethylene-propylene copolymers it is possible to determine the lengths of $-(\text{CH}_2)-$ runs based on additivity rules and comparisons with model compounds. The chemical shifts contain information on primary structure, configuration and conformation. Thus in a polymer such as polypropylene it is possible with ^{13}C NMR to get detailed tacticity information if conformational effects can be ignored. The snag is that configurational effects only arise because different configurations adopt different conformations in solution. Hence different solvents can give very different shift patterns and small compound model data can be misleading. The structure of the poly(diene) rubbers is more complicated with 1,4 and 1,2, *cis* and *trans*, and head-head, head-tail and tail-tail structures, so that the peak assignments are still not sorted out. Papers by K. J. Ivin (University of Belfast) on polycyclopentenes and polynorbornenes, and by F. Schue (Montpellier) and Y. Shabab (University of Surrey) on poly-pentadienes dealt with these aspects. Conformational effects are of great interest from the point of view of the solution and melt properties of polymers, but are in a primitive state. Cantow described attempts of his group to relate the observed temperature dependence of chemical shift to conformational energy diagrams.

While it is very nice to have detailed tacticity information, it would be good to see it put to use to further our understanding of polymerisation or polymer properties. A. Zambelli (University of Milan) showed how this can be done in experiments where, by looking at 'mistakes' in isotactic and syndiotactic polypropylenes, he was able to show that isotactic polymerisation is directed by the

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catalyst whereas in syndiotactic polymerisation it is the polymer chain which governs the orientation of a newly added unit. On occasions the results presented at the conference appeared to be a sophisticated catalogue, whereas experiments such as this present more meaty concepts.

Two papers dealt with thermosetting resins, the cinderellas of polymer science, J. R. Ebdon (University of Lancaster) on ureaformaldehyde resins and H. Pyszora (BP, Sunbury-on-Thames) on phenol-formaldehyde resins. Here NMR can only yield information about the early stages of reaction as cross linking causes loss of resolution as the molecular mobility decreases. The main lesson is that ^{13}C NMR is very useful if, as in many vinyl polymers, the tedious process of assigning peaks has already been done.

Biological polymers

Much effort in synthetic polymers is given to picking out rare events such as branches and chain ends. In proteins every group is a rare event, and the problem is first to get a resolved spectrum at all and then to get enough information to tell which peak is which. A. Allerhand (Bloomington University), Indiana showed how the non-protonated carbons of aromatic amino acids in proteins could be identified by ^{13}C NMR. These have the advantage of giving sharp peaks since they are not relaxed by attached protons, and they are spread over a wide range of chemical shift. Peaks are assigned by pH titration and chemical modification including use of bound lanthanide shift reagents. He described studies of tyrosines in myoglobins and histidines and tyrosines in lysozyme. The peaks get broader for larger proteins and high field instruments ($> 4\text{ T}$) will not improve resolution for the non-protonated carbons as chemical shift anisotropy dominates the relaxation. K. Wuthrich (STH, Zurich) showed how high field (8.4 T) proton NMR can be used to get well-resolved spectra of proteins of up to 20,000 daltons with digital resolution enhancement methods. Peaks were identified by titration, double resonance and by two dimensional Fourier transformation against chemical shift and J-coupling, to allow multiplets to be picked out. I. D. Campbell (University of Oxford) described similar experiments devised in Oxford. He showed how presaturation pulses can be used to invert and so identify multiplets and to measure exchange rates for labile protons.

These methods are most informative for proteins whose primary and X-ray structures are already known, so that amino acids can be definitely identified. Under these circumstances it is possible to study the effects of pH and ligands on structure and pick out hydrogen-

bonded groups by deuterium exchange. In denatured proteins chemical shifts for identical residues become equal so the chemical shifts in the spectrum of a native protein must contain a great deal of conformational information which cannot be interpreted as yet. A number of papers described the interaction of ligands with proteins. J. Feeney (National Institute for Medical Research, London) discussed the binding of antifolate drugs and drug fragments to dihydrofolate reductase. The effect on histidines could be observed by pH titration, and on tyrosines and tryptophans by ^{19}F and ^1H NMR of enzymes produced by bacteria fed with fluorinated and deuterated amino acids. Binding of the NADPH cofactor could be followed by ^{31}P NMR, showing a conformational change in the pyrophosphate group and that the 2'-phosphate group binds as a dianion. In a similar vein B. Lindman (Lund University) described studies of halide binding to proteins using chlorine, bromine, and iodine NMR (see Lindman & Forsen in *Nmr Basic Principles and Progress* 12, Springer Verlag, Berlin): it was possible to determine the stoichiometry of binding and, in the case of metalloenzymes, whether or not the halide binds to the metal. W. E. Hull, (Bruker, West Germany) described ^{19}F NMR of alkaline phosphatase to follow metal ion binding and ^{31}P to follow phosphate interactions. Weill described studies of cobalt and manganese in binding to polyelectrolytes by following the relaxation behaviour of the water. The overall message here seems to be that binding studies can be very informative if the NMR technique and ligand are well matched, and several independent measurements can be made. Otherwise there is often a large element of uncertainty as to interpretation.

Molecular motions

Since the advent of pulse Fourier transform spectrometers T_1 relaxation time measurements have become simple to carry out, and molecular motion studies have become an integral part of most macromolecule experiments. F. Heatley (University of Manchester) described studies of synthetic polymers in solution by ^{13}C and high field proton NMR. Measurements of correlation times from T_1 values and nuclear Overhauser enhancements disagree with one another to the extent that a correlation time distribution rather than a single time must be adopted. From proton relaxation times it is possible to show that a χ^2 correlation time distribution cannot fit the data over a wide temperature range, but that a molecular jump model can be used in combination with overall molecular rotation. These models seem to have great potential and are based on an enumeration of the possible conformational changes of chain segments with

fixed ends. For a diamond lattice, for instance, these jumps involve motions of three and four bonds simultaneously (see Valeur *et al.* *J. Polymer. Sci. (Physics)* 13, 667, 675; 1975).

N. Gronski (Freiburg University) and H. C. Broecker (University of Hamburg) used such models for polydienes and polyisobutylene, and F. Laupretre (Paris) for polystyrene. One criticism of this work is that there is little effort to compare the results with those from dielectric and other relaxation methods. On the biopolymer front T_1 values are extensively used to measure exchange processes and to look at phenyl group rotations, though in this latter case, it is hard to distinguish between full rotation and oscillations (see *News and Views* 258, 112; 1975). This is an area where we can expect a lot of progress and consequently an increase in our knowledge of conformational flexibility of proteins in solution. High resolution NMR of solid polymers using a combination of high power proton decoupling and magic angle spinning was described by J. Schaeffer (Monsanto, St Louis) who related the correlation time data to the mechanical properties of the polymers (see *News and Views* (1976) 261, 457; 1976 and *Macromolecules* 10, 384; 1977).

NMR of polymers has often been accused of never generating indisputable information. This is becoming less true, particularly if the applications are carefully chosen. The advantage of NMR over other techniques is that resonances from individual groups are observed and can be monitored to give detailed information about their states (motional, configurational, conformational, ionisation). A disadvantage is the limited frequency range available which often can make the results ambiguous. For enzyme studies a further disadvantage is that even for ^1H NMR relatively large amounts of sample (0.5 ml of 0.5 mM solution) are required. □

Neoplastic transformation

from N. A. Mitchison

The Dahlem Conference on Neoplastic Transformation was held in Berlin on 9-13 May, 1977, and was organised by Dr Silke Bernhard. The proceedings will be obtainable from Dahlem Konferenzen, Delbrückstrasse 4c, D-1000 Berlin 33.

THE conference aimed at assessing current knowledge and tried to pose questions that would draw attention to gaps in our understanding of the pro-

cess by which normal cells become tumours. One was left with the vivid impression that cancer research continues to provide an area of excitement, in which much of what is new in biology comes up for scrutiny. An account of the proceedings will be published within six months. The participants mentioned in this report are not necessarily, of course, the authors of the views which they discuss.

Epidemiology was much discussed. Interest is at present greatest in environmental causes of cancer, having moved from previous concern with radiation and viruses. The strongest view (Muir) is that as much as 80% of cancer may be caused by environmental agents. The evidence comes partly from direct study of the effects of tobacco and alcohol, which are known to cause about 40% of cancers in Western males, and partly from study of particular industrial hazards, known to cause at least 1%. Comparisons between different environments tend to support the environmentalist view; the difference in cancer incidence between Mormons and their fellow US citizens, for example, is especially striking (C. S. Muir, International Agency for Research on Cancer, Lyons; R. W. Miller, National Cancer Institute, Bethesda). On the other hand it can be argued that all cancers are 100% genetic, just as they are also 100% environmental in origin. And although early pregnancy protects statistically against cancer of the breast, J. Cairns (Imperial Cancer Research Fund, London) pointed out that one cannot usefully describe the avoiding of pregnancy as an environmental cause of cancer.

Widespread use of the Ames test has confirmed the mutational origin of cancer (A. G. Knudson, Institute for Cancer Research, Philadelphia). The monoclonal nature of most tumours supports this view (P. J. Fialkow, Veterans Administration Hospital, Seattle); the exceptions to this rule are understandable, for example, hereditary multiple neurofibroma or infective warts. So also does the predisposition to cancer in inherited defects in DNA repair, particularly xeroderma pigmentosum and ataxia telangiectasia. Somatic genetics has now reached the point where the first of these diseases can be subdivided into six complementation groups, and the second into three. Yet further support for the mutation view comes from the biochemistry of DNA repair: the failure to excise O⁶-ethylguanine explains why ethyl nitrosourea causes tumours in the rat brain

(K. Rajewsky, Universität Köln). We are left with two main questions: how important are endogenous carcinogens; those detected by mutational screening of bile or urine (P. N. Magee, Temple University, Philadelphia) for example, and do environmental carcinogens act as promoters rather than as initiators (Cairns)?

How do viruses transform cells (W. Doerfler, Universität Köln; E. L. Winaker, Universität München; C. M. Croce, Wistar Institute, Philadelphia)? The nearest we can come to answering this question at present is to find out whether SV40 or the RNA leukaemia viruses integrate into a definite site in the host genome, presumably adjacent to the oncogene(s). The evidence is still inconclusive. Cutting the genome of SV40-transformed cells with restriction enzymes suggests that integration is promiscuous; but cell hybridisation studies indicate that integration occurs preferentially in human chromosomes 7 or 17 (Croce). Further analysis using restriction enzymes should answer the question.

A respectable case can be made for the proposition that cancer results not from a stable genetic change, but from a reversible form of differentiation. Thus, in chimaeric mice, genetic markers from teratomas have been detected in normal, differentiated tissue (R. L. Gardner, University of Oxford); however, the most dramatic finding, by B. Minz, that teratoma-derived gametes can give rise to normal offspring, has yet to be confirmed. Since at least nine different chemical factors control the growth of cells in vitro (C. H. Sato, University of California, San Diego)—and the list is rapidly growing—there is plenty of scope for control of differentiation. However, the decisive experiments have proved curiously elusive: nuclear transplantation, or the isolation of normal cells from tumours other than teratomas.

Cancer immunology survives, in spite of the collapse of immunological surveillance as a general theory. Indeed, surveillance probably does operate, but only in restricted circumstances involving viral antigens such as nasopharyngeal carcinoma of the Chinese. Up to date cellular immunology links up with cancer when the question is posed whether manipulation of the immune system may reveal otherwise undetectable responses to weak tumour antigens (B. A. Askonas, National Institute for Medical Research, London; Rajewsky; K. P. Eichmann, Institute für Immunologie und Genetik, Heidelberg). The idiotypic network, and T helper cells, provide logical handles for such manipulation. The most exciting development in cancer immunology has been

the discovery that tumour specific antigens and major histocompatibility antigens interact in several ways (E. S. Lennox, MRC Laboratory of Molecular Biology, Cambridge). Thus new H-2 specificities occur on cancer cells, as a result either of derepression or post-translational modifications. This implies that the molecular biology of the tumour specific antigens may be more accessible than had been expected. □

Snake's eye view of Adam and Eve

from K. W. Jones

A MOLECULAR difference at the DNA level between human males and females (surely fuel for the current debate on whether or not man is man or merely person) is the substance of recent work by Kunkel *et al.* (*Science* **191**, 1189; 1976). Males possess a Y chromosome which is absent from normal females and carries the gene(s) which determines primary sex. By reassociation of traces of male DNA with an excess of female DNA these workers have isolated a class of repeated DNA (It-Y DNA) which does not reassociate with female DNA, but which will reassociate with male DNA. It is therefore presumably of Y chromosome origin. More recently (*Proc. natn. Acad. Sci. U.S.A.* **74**, 1245; 1977) the same workers have analysed the content of It-Y DNA in the DNA from individuals with various structural abnormalities of the Y chromosome and shown that It-Y DNA comprises 15–30 different families of reiterated sequences, is largely located on the fluorescent part of the long arm of the Y and amounts to about 10% of the total chromosome. This constitutes the first characterisation of repeated DNA unique to a single human chromosome. The fact that it is on the Y chromosome immediately poses the question of its role in sex determination but, as the authors point out, such a role is rather unlikely since in their study some normal males with small non-fluorescent Y chromosomes lacked It-Y DNA whereas some females with Y translocations did possess it.

Recently Cooke (*Nature* **262**, 182; 1976) has described repeated DNA specific to human males and amounting to 50% of the Y chromosome. This was detected by digesting DNA from males and females with the restriction enzyme *HaeIII* which cuts DNA at a short defined base sequence to yield fragments of different lengths defined in distribution according to the spacing of the target sequence cleaved by the enzyme. When the fragments were

separated by gel electrophoresis in agarose, DNA bands were seen in the male digest which were absent from the female digest, suggesting the unique presence of a repeated sequence containing the *Hae*III target site in male and therefore in Y chromosome DNA. Since this large fraction of Y chromosome DNA is not included in the It-Y DNA recovered by Kunkel *et al.*, it must now be supposed that it is sufficiently homologous with DNA present in the female to be removed by re-association analysis. From my own observations, Cooke's DNA fractions are located essentially in the fluorescent region of the Y along with the It-Y DNA.

Highly repeated simple sequence (satellite) DNAs in man are also concentrated in the fluorescent long arm of the Y as well as being widely represented in many other human chromosomes. The relationship of the satellite DNAs to the other Y-repeated DNAs is not known at present and their quantitative contribution to the Y DNA as a whole is also not determined. It seems however that most of the Y chromosome long arm is composed of repeated DNA and that of this, only It-Y DNA is unique to this chromosome. The implication of the finding that It-Y DNA maps to the fluorescent long arm of the Y is that the entire repeated DNA of the remainder of the chromosome, including that in the region which contains the sex-determining gene(s) is sufficiently homologous with DNA present elsewhere in the genome to be included in the fraction which reassociates with female DNA in the conditions of Kunkel *et al.*'s experiments. This is perhaps not too surprising since the X and Y chromosomes were presumably homologous at some early period in their evolution, and it has been suggested that they are derived by a process of translocation from structural homologues (see Lyon *Nature* **250**, 651; 1976).

The significance of the high concentration of repeated DNA on the Y chromosome or indeed of the significance of most non-transcribed repeated DNA is unknown. According to Miklos and Nankivell (*Chromosoma* **56**, 143; 1976) satellite DNA may play a part in controlling the frequency of crossing over between homologous chromosomes. By crossing over, pairs of chromosomes remain largely homologous through the frequent interchange of DNA. Conversely, chromosomes which fail to cross over will tend to evolve divergently. Some change inhibiting crossing over would therefore seem to be a primary requirement for

the evolution of specialised sex chromosomes.

Snake's eye view

According to Singh *et al.* (*Chromosoma* **59**, 43; 1976), in the snakes, which exhibit the entire process of evolution of sex chromosomes, the first discernable change is in the repeated DNA structure of one of a pair of chromosomes bearing the sex determining gene(s). Within the snakes there is a range of forms from primitives with no defined sex chromosomes through intermediate forms with definite, though morphologically identical, sex chromosomes, (called ZW in the female, which is heterogametic, and ZZ in the male which is homogametic) to forms with highly differentiated, morphologically dissimilar Z and W chromosomes. The primitive snakes show no difference between male and female in highly repeated (satellite) DNA whereas the intermediate forms show very obvious differences involving the appearance of novel satellite DNA in the female. This novel DNA maps exclusively, by *in situ* hybridisation, to the W chromosome (which is the functional equivalent of the Y in mammals) where it is spread over the entire length. The identical-looking Z chromosome lacks this DNA. The W chromosome also shows a different cycle of DNA synthesis from the Z, as is frequently the case in other satellite-DNA-rich chromosomal regions. Singh *et al.* suggest that this interferes with pairing between the Z and W thus predisposing to their divergent evolution and accounting for the fact that in the most evolutionarily advanced group of snakes the W (like the Y in man) has become greatly reduced in size and modified in form relative to the Z. The modified W chromosome nevertheless has conserved the satellite DNA sequences found in the W of the intermediate group, as determined by molecular hybridisation, as well as its allocyclic DNA synthesis pattern. From this it may be suggested that in the human Y chromosome the plethora of simple sequence DNAs reflects the molecular end consequences of the mechanism which played a part in its specialisation. Once a chromosome, like the Y, is genetically isolated, the constraints on acquiring, or amplifying, additional non-transcribed (functionless) DNA sequences are probably lost. The Y chromosome is therefore likely to be loaded with "junk DNA" (Ohno *Hoechst Symposium*; 1972) which no longer serves a useful purpose. At a practical level, however, it now seems a feasible and exciting prospect to use the It-Y DNA as a handle to fish out covalent Y chromosome DNA sequences by some type of DNA affinity

chromatography. This will shed further light on the structure and evolution of the human Y chromosome and it may ultimately permit a direct approach to the molecular basis of sex determination at the DNA level. □



A hundred years ago

IN the debate on the education estimates, on Tuesday night, Sir John Lubbock, speaking on the extra subjects which had been made compulsory, said he doubted whether under any circumstances it would be desirable thus to stereotype one form of education for the whole of England; but surely we ought not to do so unless we were very clear as to what is the best system. There was, however, very great difference of opinion on this head. The first authority to which he would refer was that of a committee of that House. It was presided over by his hon. friend the member for Banbury, and after careful enquiry they reported that in their opinion "elementary instruction in the phenomena of nature should be given in elementary schools." The next authority which he would quote was the Royal Commission, presided over by the Duke of Devonshire, which unanimously recommended that more substantial encouragement should be given to the teaching of the rudiments of science in our elementary schools. In Scotland, too, great dissatisfaction was felt with the present system. At the last conference of elementary teachers, held in London, which was very numerously attended, it was resolved that the system of payment "embodied in the Code is unsound in principle and injurious to the progress of true education." The inspectors of schools differed greatly as to the most suitable subjects. Even in regard to geography they were not unanimous. It was said as a subject to lend itself very much to "cram." One of the inspectors gave in support some very amusing answers. For instance, in answer to a question of "What are mountains and rivers?" one girl replied "Mountains in some parts of the world are very useful. In Africa, for instance, they shoot out gold." Of rivers she had not so favourable an opinion, though she thought "they were all very well in some countries where there was very little rain." He confessed, however, that he thought geography a very good subject, though he was not convinced that it ought to be continued during the whole course to the exclusion of other subjects. The mere skeleton of history taught in our elementary schools contained little more than dates, wars, and murders; but dates and crimes no more constituted the history of a nation than sinews and bones made a man. Men of science must be grateful to Sir John Lubbock for so constantly urging upon Government the importance of scientific education.

From *Nature* **16**, 12 July, 216; 1877.

review article

Transposable genetic elements as agents of gene instability and chromosomal rearrangements

Patricia Nevers & Heinz Saedler*

Transposable genetic elements in prokaryotes and eukaryotes, when inserted at a given locus, can control expression of the locus and cause large scale rearrangements of adjacent DNA sequences. Striking similarities in genetic behaviour between the two groups of elements have led to the proposal of a molecular model of eukaryotic controlling elements, and to suggestions about the part such elements may play in evolution and differentiation.

CHROMOSOMES are commonly regarded as conservative structures in which an exact amount of genetic information is arranged in a definite sequential order. This order is normally preserved when information is exchanged between chromosomes, and guaranteed by a set of recombination enzymes that function only with paired sectors of homologous DNA. But processes such as inversion, deletion, duplication, and translocation, often involving recombination between apparently non-homologous chromosomal regions, can alter the sequence of genetic information. The resulting chromosomal rearrangements have been observed in some cases with a disturbingly high frequency, inviting speculation about their biological significance. The processes involved have been termed collectively 'illegitimate recombination',^{1,2} reflecting the bias for conventional pathways based on sequence homology; but evidence now accumulating may legitimise them as important mediators of evolution or even differentiation.

Classical geneticists have known for decades that eukaryotic chromosomes can behave unconventionally, giving rise to unexpected changes in phenotype. In the somatic tissue of plants or fruit flies, random spots of pigment arise against a background that is otherwise colourless, resulting in a mottled or variegated appearance. Closer examination of this phenomenon in maize led Barbara McClintock to the postulation that distinct genetic units—so called 'controlling elements'—are responsible for such changes in gene expression³. Drastic alterations of chromosome organisation, such as deletions, inversions and duplications, were often found in association with loci containing controlling elements⁴. Moreover, a given controlling element did not remain fixed at a particular site in the chromosome, but seemed to be capable of moving from one position to another, causing instability of gene expression and structural alterations at each new locus⁵⁻⁹.

Thus controlling elements were revealed as possible agents of illegitimate recombination. But, as with many discoveries in classical genetics, interest in these elements was confined to a small group of scientists until molecular biologists succeeded, fifteen years later, in isolating analogous elements, the insertion sequences (IS), in prokaryotes¹⁰⁻¹².

The properties of IS-elements in bacteria and controlling elements in *Zea mays* have been described in detail elsewhere^{9,13}. The aim of this article is to outline briefly what is known about bacterial insertion sequences at the molecular level, concentrating on the elements IS1 and IS2; to draw attention to parallels between the elements of eukaryotes and prokaryotes; to interpret data on eukaryotic controlling elements in the form of a molecular model; and to suggest what part these elements may play in evolution or differentiation.

IS elements in bacteria

Studies on the galactose and lactose operons of *Escherichia coli* revealed an unusual group of mutants that were shown conclusively to be caused by the insertion of a segment of DNA into the operon (for review, see ref. 13). When integrated in a gene of an operon, a DNA sequence of this kind not only abolishes the function of the gene, but also leads to a reduction in the activities of all other genes downstream in the direction of transcription, an effect known for historical reasons as polarity. (The term was originally used in connection with 'polar' mutations, in which the expression of genes downstream of the mutation is inhibited. Although it is now known that it is transcription that is polar, the mutations merely interfering with its progress, they are still said to 'cause polarity')¹³⁻¹⁷. The insertion sequences investigated so far fall into five non-homologous classes: IS1, IS2, IS3, IS4 and IS5 (for review, see ref. 18). The small size of these elements (800-1,400 nucleotide pairs), equivalent to only one or two genes, indicates that they differ from any known bacteriophage, and indeed, they seem to represent material displaced from the bacterial chromosome rather than invaders from without¹⁹⁻²¹. Eight copies of IS1 and five of IS2 are found as natural residents of wild-type *E. coli* chromosomes. Examination of their properties at 'unnatural' positions in the galactose and lactose operons has been conducted in order to gain information about their possible biological significance as components of wild-type chromosomes.

Polar effects of IS-sequences

The polar effects of insertion sequences have been shown to be due to interference at the level of transcription²². While the physical presence of an element in an operon causes some reduction in transcriptional read-through, a maximal block

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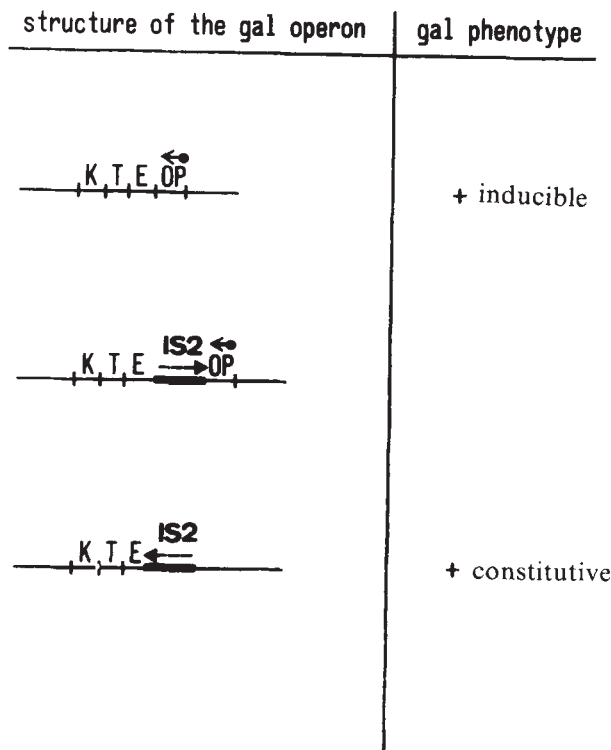


Fig. 1 Control of gene expression by IS2. Expression of the three genes of the galactose operon of *E. coli*—galactokinase (K), transferase (T), and epimerase (E)—before the insertion of IS2 is shown in line one. The genes are under normal operator/repressor control and expressed only in the presence of an appropriate inducer. Integration of IS2 into the operator region in polar orientation abolishes operon expression (line 2). Integration of IS2 in the opposite orientation leads to constitutive expression of the operon³⁸. Long arrows indicate the orientation of IS2 and short arrows the direction of transcription from the operator region.

is achieved through the action of the bacterial protein rho²³⁻²⁷. According to a recent model of normal transcription termination in bacteria and phage²⁸, the rho protein recognises specific DNA sequences, the terminator sequences, at which it stops transcription. However, in order to be effective, rho must react with ribosome-free mRNA at these sites. This condition could be met if so-called 'nonsense' codons are situated immediately ahead of a terminator site. At these codons, for which no corresponding amino acids exist, translation ceases and ribosomes are released from the mRNA²⁹. Thus it is possible that IS elements contain a combination of nonsense and terminator sequences which lead to rho dependent termination of transcription.

Excision of IS-elements

Wild-type revertants of IS-induced mutations, in which the IS-element is missing, can be found with a frequency of one in ten million^{10,23}. They are thought to arise by exact excision of the IS element. Since normal function of the gene is restored, the integration and excision of the element must be possible without damage to the gene. But such precision is not always the rule, and in some cases, integration or excision is accompanied by deletions of bacterial material adjacent to the insertion site^{13,30,31}. The enzymes involved in integration and excision have not yet been identified, but they are independent of known recombination enzymes, since both events occur in rec A hosts^{14,32}.

Transposition of bacterial DNA insertion elements

The excision of an IS sequence followed by re-integration of the element at another site is termed transposition. This process has been examined most extensively with transposons—elements

closely related to IS sequences and which have been the subject of a recent review³³. A transposon is a length of DNA embracing several genes and bordered at each end by identical sequences in reverse or direct orientation to each other. In a number of cases these termini consist of known IS sequences, while the intermittent material may include genes for resistance to antibiotics, positive markers which can be used to monitor the movements of the transposon. Transposons have thus been shown to move from the R (resistance) plasmid, in which they normally reside, to other plasmids such as the F (fertility) plasmid, to the bacterial chromosome, and to various bacteriophage DNAs as well (for review see ref. 33).

Transposition may be mediated by enzymes that are able to recognise the termini of insertion sequences and certain bacterial sequences. Alterations of these sequences, such as deletions, may influence the efficiency of transposition, as shown with the ampicillin transposon TnA (ref. 34). Transposition occurs in rec A hosts³⁵⁻³⁷, which indicates that transposition enzymes are also independent of normal recombination functions. In one case, that of TnA, the transposon itself seems to code for transposition functions³⁴.

Control of gene expression by IS2

It seems that insertion sequences may be the components of a unique system of negative control, eliminating gene and operon activity on integration (see section on polar effects) and restoring it through precise excision. But from an evolutionary point of view a more interesting feature of IS elements may be their ability to implement positive control, as illustrated by the element IS2 (Fig. 1). While IS1 reduces operon expression when integrated in either direction, IS2 does so only when integrated in a particular orientation, orientation I. When it is in the opposite orientation, orientation II, genes located downstream are expressed constitutively at a level three times higher than normal³⁸. This has been shown to be due to the presence on IS2 of a promoter at which transcription can be initiated³⁹. This promoter may have other consequences when IS2 is integrated in orientation I, when transcription from the IS2 promoter may run head-on into an RNA polymerase coming from the existing promoter of the operon, thus contributing to the inhibitory effect of IS2 in orientation I (ref. 40).

The two antipodal effects of IS2 suggest that it can act as a simple switch. This is particularly appealing in view of evidence suggesting that 'flip-flop' control mechanisms underlie phenomena such as the alternating production of two different types of flagella in *Salmonella*⁴¹⁻⁴³ or the determination of mating type in *Schizosaccharomyces*⁴⁴. An essential feature of such a switch mechanism would be the inversion *in situ* of IS2 or an equivalent element. There is genetic evidence for the inversion of IS2 in *E. coli*³⁸, although it has not yet been demonstrated by physical means. That inversion may represent a fundamental mode of regulation is shown by data from the bacteriophage mu. Inversion *in situ* of the so called 'G'-loop in this phage is clear both from genetic and from physical evidence^{45,46}.

IS sequences involved in chromosomal rearrangements

As striking as IS control of gene expression are the chromosomal rearrangements found in association with some insertion sequences. One prominent example is that of IS1-mediated deletion^{31,47}, illustrated in Fig. 6a. Sequences adjacent to this element are deleted with a frequency 10²–10³ times greater than the spontaneous frequency. In the cases investigated so far, IS1 is left behind in the chromosome during this process and continues to cause secondary deletions. IS1-mediated deletions all start at one terminus of the element and extend into the chromosome, ending at various non-random sites so that different classes of deletions occur with characteristic frequencies. Here too rec A-independent enzymes capable of recognising an IS terminus and certain chromosomal sites seem to be involved. Preliminary steps towards the identification of these enzymes have been made by the isolation of a bacterial mutant

in which IS1-mediated deletion is reduced fortyfold⁴⁸. Interestingly, the frequency of IS1 excision is not affected in the mutant, which suggests that different sets of enzymes are involved in these two processes.

Another type of rearrangement, duplication, can theoretically arise if chromosomal material is deleted in the form of a transposable structure which moves from one daughter strand to the other during replication. Duplications in association with an IS-element have been observed in mutants harbouring IS2 in the operator region of the galactose operon⁴⁹.

Controlling elements in *Zea mays*

In general, the effects of controlling elements in maize parallel those of DNA insertions in prokaryotes: integration of an element into a locus may produce a mutant phenotype, while accurate excision in some cells leads to clones in which normal expression of the locus is restored. If the locus at which an element is integrated contributes to a visible phenotype, such as pigmentation, occasional excision of the element during development results in a variegated appearance. In maize two kinds of element can be distinguished^{9,50}: entities with no known gene products, referred to as receptor elements, and more complex, trans-acting units called regulatory elements. These elements can undergo integration, excision and transposition, for which specific enzymes may be postulated, as with bacterial DNA insertions. In maize, however, activities corresponding to such enzymes are supplied by the regulatory element itself, in a manner analogous to the bacterial TnA transposon (see section on transposition of DNA insertions). A regulatory element can thus move autonomously and govern the movements of receptor elements as well. The combination of a trans-active regulatory unit and a non-autonomous receptor

element constitutes a two-element system, several of which have been found in maize, including the systems activator-dissociation (modulator), dotted, and Spm (enhancer).

These systems are entirely independent of each other, exhibiting no cross reactions between the regulator functions of one system and the elements of another. A detailed review of controlling elements in maize has been presented in a recent article⁹.

For the following discussion we have selected Spm (suppressor-mutator), equivalent to En (enhancer) as a representative system, since it has some unusual properties not seen in other systems and has been the most thoroughly investigated. At this point it should be noted that most of the evidence for controlling elements has been obtained by classical genetic means. We shall waive a comprehensive presentation of this evidence and offer an interpretation on a molecular basis instead, in the hope of encouraging more widespread interest in this subject.

Integration of an Spm element at the A_1 locus

The A_1 locus on chromosome 3 of *Zea mays* is one of a number of loci that contribute to anthocyanin production. As with most eukaryotic loci, the exact molecular nature of A_1 is unknown, but it may embrace several structural genes together with extensive control regions. Accordingly, various kinds of mutant can result, depending upon the position in the locus into which an element is inserted. For example, integration into a structural gene could lead either to total loss of the corresponding gene product or to loss of its distal end, resulting in a modified product. An element situated between a gene and its promoter may block transcription completely, allow a reduced level of expression, or have no effect at all.

In the mutants a_1^{m-5} (ref. 51) and the original a_1^m (ref. 52), the regulatory unit of the Spm system, referred to simply as Spm, is integrated in the A_1 locus. Anthocyanin production is eliminated in a_1^{m-5} and a_1^m kernels, but frequent, self-induced excision of the elements during plant growth results in pigmented sectors (Fig. 2, rows 2 and 3).

Trans-active functions of Spm

In the mutant a_1^{m-1} , as opposed to those discussed above, only the receptor element of the Spm system is incorporated in the A_1 locus³. In the original isolate of this mutant, expression of the A_1 locus is barely affected, and kernels exhibit a relatively high level of homogeneous pigmentation in the absence of Spm regulatory activity (Fig. 2, row 4). When an active Spm is present at some other position in the genome of a_1^{m-1} kernels, a different phenotype is produced. In these kernels, containing both elements of the Spm system, background coloration is abolished completely (Fig. 2, row 5). This reflects a unique property of the Spm regulatory element, which produces a suppressor activity that interacts with the receptor element at the A_1 locus to eliminate A_1 expression in the background tissue of the kernel. However, pigmented spots arise through excision of the receptor element from the A_1 locus under the influence of a second Spm activity, the mutator. The two trans-active Spm functions, suppressor and mutator, seem to be able to undergo mutation independently^{53,54}, which suggests that they are coded by separate genes on Spm.

States of a locus

In addition to mediating excision, the mutator product of Spm can induce inheritable alterations of the receptor element, leading to new 'states' of the locus in which it resides^{54,55}. For example, exposure of the receptor element in a a_1^{m-1} plants to Spm mutator activity leads to derivatives with new properties, representing different 'states' of the a_1^{m-1} locus^{53,54} (see Fig. 2, rows 4-7). Some states derived from a_1^{m-1} show new levels of homogeneous pigmentation in the absence of Spm. Other states, derived from the allele, a_2^{m-1} , (ref. 56) respond to the suppressor but not the mutator activity of Spm, or vice versa. Still other, stable derivatives with a mutant phenotype respond

Fig. 2 States of the A_1 locus in *Zea mays* and influence of Spm on gene expression. In wildtype kernels, in which no controlling element is present at the A_1 locus (row 1), anthocyanin production and pigmentation are maximal and independent of the presence of Spm elsewhere in the genome. The degree of background coloration in all other kernels indicates the expression of the A_1 locus as a function of Spm activity. Black spots correspond to clones in which a controlling element has been excised from the A_1 locus under the influence of Spm mutator activity. The white spots on the kernel in row 9 are due to inactivation of Spm in two cells and their progeny. (For further explanation, see text).

phenotype of kernel	A_1 locus	state	Spm activity	location of Spm
	A_1		+ and -	not at A_1
	$a_1^{m(-5)}$		-	A_1
	$a_1^{m(-5)}$		+	A_1
	a_1^{m-1}	1	-	none
	a_1^{m-1}	1	+	not at A_1
	a_1^{m-1}	2	-	none
	a_1^{m-1}	2	+	not at A_1
	a_1^{m-2}	1	-	A_1
	a_1^{m-2}	1	+	A_1

Structure and function of active Spm

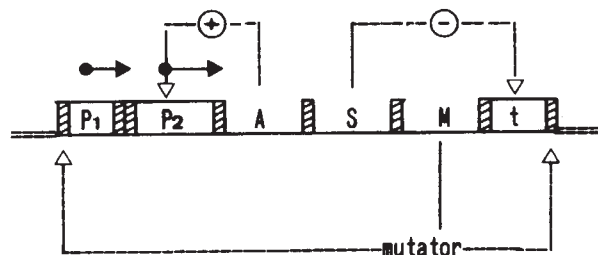


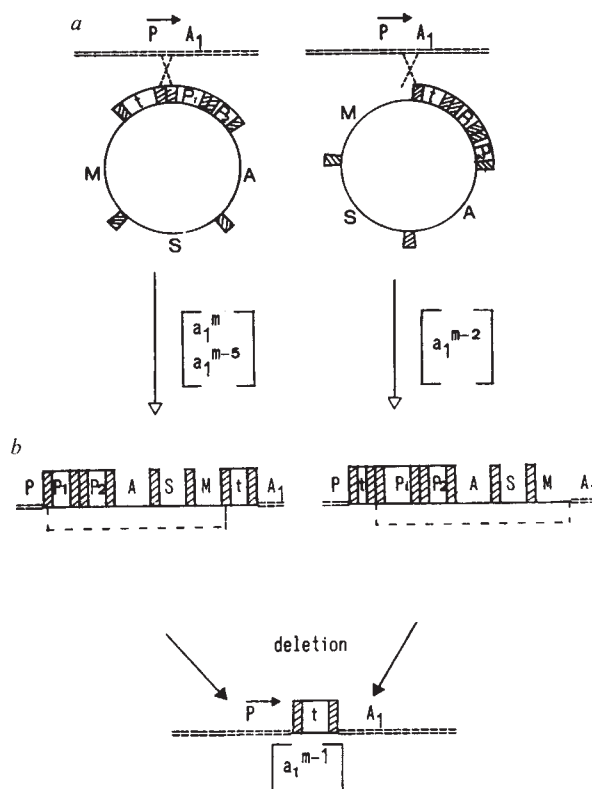
Fig. 3 Proposed structure and function of active Spm. A model of the molecular structure of an integrated Spm regulator element is illustrated. At one end of the element are two units, each with a promoter, P1 and P2. The direction of transcription from these promoters is indicated by the arrows above them. At the other end of Spm is a unit with a terminator sequence, t. Three structural genes are included in the element, coding for the A (activator), S (suppressor), and M (mutator) products. The action of these products is depicted by the dashed lines. The suppressor product operates at the t sequence to terminate transcription, as indicated by the symbol $\ominus$. The activator product, on the other hand, has a positive effect on transcription ($\oplus$), by activating the P2 promoter. The mutator function of Spm is involved in excision of the element at the terminal sequences. So-called 'black box sequences', at which recombinational events such as inversion, deletions and duplications can occur, are represented by vertical hatched bars.

unit similar to IS1, with a terminator sequence. Transcription can start spontaneously from P1, but transcription from P2 requires activation by the A gene product. The suppressor product of the S gene is envisaged as causing termination at the terminator sequence. The mutator product of the M gene executes excision at the indicated sites and induces alterations leading to new states, as discussed above. At the ends of Spm and interspersed throughout the element at various junctions are 'black box sequences', representing repeated sequences in reverse or direct orientation, comparable with those at the ends of bacterial transposons³⁷. These sequences are not necessarily identical, but denote sites at which recombinational events such as inversions or deletions can occur.

Different modes of integration of Spm

By analogy with models of equivalent transposon processes, transposition of Spm can be considered as beginning with excision at the terminal sequences of the element, followed by circularisation and re-integration, as shown in Fig. 4(a). This process is catalysed by the mutator product. Integration at the site on the circular element between P1 and t gives mutants corresponding to a_1^{m-5} and a_1^m , in which the A locus is no longer expressed. Occasionally integration may take place at a different site on the circular intermediate, for example, the junction between M and t. This generates a mutant in which transcription started at the Spm promoter continues into the A1 locus. In this case, contrary to most situations described so far, the active element does not prohibit expression of the locus but promotes it. Obviously such mutants may be barely distinguishable phenotypically from wild type. However, if Spm is not transcribed, as in inactive phases described below, the mutant character is revealed, since no pigment is formed. This is exactly the case of the original mutant a_1^{m-2} (ref. 53) (Fig. 2, rows 8 and 9), which has been crucial to considerations of the molecular structure of Spm.

Fig. 4 a, Integration of Spm at the A1 locus in chromosome 3 of *Zea mays*, producing the mutant alleles a_1^{m-5} , a_1^m , and a_1^{m-2} whose phenotypes are depicted in Fig. 2. **b**, Possible origin of the receptor element from the alleles depicted in 4(a), leading to a two-element system.



to neither Spm activity. One explanation is that new states arise through recombinational events such as inversions, deletions and duplications, or through conventional mutational events within the receptor element, altering its capacity to obstruct transcriptional read-through or the target sites for suppressor and mutator action. Short-range transposition of the receptor element within the locus may also be involved in some cases.

Transposition of controlling elements

A striking characteristic of controlling elements is their ability to move from site to site in the genome. Transposition of Spm, involving excision from one position and re-integration at another, is controlled by the mutator product of Spm, which seems to be effective only in the presence of suppressor activity⁵⁴. Thus an active Spm can and does transpose itself autonomously, while an Spm that is inactive, as discussed below, remains fixed at one position.

While excision of the receptor element of the Spm system is often seen, transposition has been reported in only one instance, that of the mutant wx_2^{m-8} ($wx = waxy$) derived from an a_2^{m-1} plant⁵⁷. It should be noted, however, that transposition of the receptor element is common in other two-element systems in maize³.

A model of Spm structure and functions

The repertoire of Spm activities is by no means exhausted by those described above. But before proceeding to a series of more subtle phenomena, such as cyclic changes in Spm activity and trans-activation, we wish to introduce a molecular model of Spm based on our knowledge of IS elements. It has been designed to fit a variety of observations into an intelligible framework and to promote discussion of new experiments.

The proposed molecular structure and functions of Spm are depicted in Fig. 3. The element includes three structural genes, one each for the suppressor and mutator products discussed above, and a third coding for a positive regulatory function, the activator, which will be discussed in the section dealing with trans-activation. There are two units analogous to IS2 at one end of the element, each with a promoter. At the other end is a

Origin of the receptor element

Having established a model for Spm and mutants such as a_1^{m-5}/a_1^{m-1} and a_1^{m-2} caused by the integration of Spm into A1, let us now consider the origin of the receptor element in the Spm system. In a number of cases^{7,51,52,53}, a mutant harbouring a single, autonomous regulatory element at a given locus has generated derivatives in which only the receptor element is found at the locus, apparently accompanied by transposition of the regulatory element to another position. An explanation for this observation, which has been discussed elsewhere⁵⁸, is elaborated in Fig. 4(b).

When part of an integrated Spm is deleted, perhaps through inaccurate excision, the t unit may be left behind as a fragment that is still responsive to Spm, since it contains the target sites for both the suppressor and mutator functions. This is the situation found in the mutant a_1^{m-1} discussed earlier. When this residual unit is located between an A₁ structural gene and its promoter, transcriptional read-through may be reduced to some extent. However, when suppressor product is supplied by an active Spm, it acts as the terminator sequence of the receptor element to block transcription completely, just as the bacterial rho protein does at IS-sequences. The original level of expression of the locus can be restored when the receptor element is excised.

In addition to producing a receptor element corresponding to a complete t unit, as discussed above, partial deletion of Spm could also leave behind fragments that are greater or smaller than the t unit, resulting in various different states of the locus⁸.

It is especially significant that the exceptional mutant a_1^{m-2} can generate a two-element system with a phenotype similar to the a_1^{m-1} phenotype described above⁵³. This lends support to the proposal that the regulatory element in a_1^{m-2} mutants contains the same components as in other mutants such as a_1^{m-5} but in a different order, as shown in Fig. 4(a).

Cyclic changes in activity of Spm

An interesting property of Spm is its tendency to become inactive^{54,59}. Inactive phases alternate with periods of activity with a characteristic frequency. Since an inactive element, expressing neither of its two products, occasionally becomes reactivated, these products do not seem to participate in such an activity change^{54,60}.

The effector of cyclic activity changes must be sought elsewhere, as shown by mutant alleles such as $a_1^{m(f1ow)}$ (ref. 59), which exhibit Spm activity only in certain parts of the kernel. The mutant $a_1^{m(f1ow)}$, has an Spm subject to activity changes at the A1 locus. In kernels of this mutant Spm is active only within a limited area at the base of the kernel. The element in the $a_1^{m(f1ow)}$ mutant is apparently sensitive to activating signals produced during the development of the base of the kernel.

In the terms of the molecular model of Spm, cyclic activity changes may result from inversion *in situ* of the promoters under the influence of extraneous signals, as in the inversion of IS2 discussed earlier.

Inversion of one or both promoters produces three different inactive structures, shown in Fig. 5. The result of inverting both promoters (Fig. 5a) is obvious. When only P2 is inverted, as in Fig. 5(b), RNA polymerase can still initiate transcription at the P1 promoter, but it will be obstructed if it collides with transcription machinery from the P2 promoter (see section on polar effects of IS2). When only P1 is inverted, as in Fig. 5(c), transcription of Spm from P2 can continue as long as activator product is present in the cell. The element will become inactive only when activator product in the cell has been sufficiently diluted. Because of this dilution requirement, it is unlikely that inversion of P1 is involved in somatic activity changes that occur during the development of a kernel. But an inactive structure of the kind shown in Fig. 5(c), is important for considerations of trans-activation, discussed in the next section.

Through the action of mutator product, an Spm regulatory

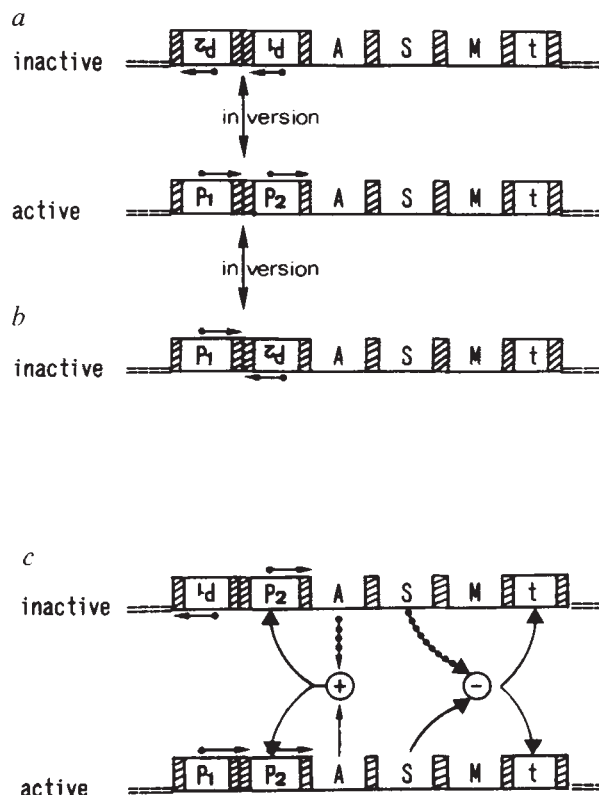


Fig. 5 Cyclic changes in activity of Spm. Inversion of one or both Spm promoters leads to three different inactive structures (a, b and c). The structure illustrated in c can be trans-activated when activator product is supplied by an active Spm.

element that exhibits a certain frequency of cyclic activity changes can mutate to alternate with a new frequency⁶⁰. This indicates that something within the element itself determines the frequency (and time) of activity changes. These determinants are represented by the 'black box' sequences shown in the model in Fig. 3, at which inversion might occur. Mutational alteration of these sequences could affect the frequency of inversion.

Trans-activation of Spm

In a key experiment it has been demonstrated that an inactive Spm can be temporarily activated when a second fully functional Spm is introduced into the genome⁶⁰. This was detected in a unique set of conditions which made possible phenotypic discrimination between kernels with double Spm activity and those with only one dose.

Normally when a receptor element is integrated at a locus, the same phenotype is produced regardless of whether one or more Spms are present in the genome. In one experiment, however, a special allele of a_2^{m-1} was used to test for Spm activity⁵⁵. In plants with this allele the receptor element at the A₂ locus allows some pigment production when Spm is absent. In the presence of Spm suppressor product expression of the locus is eliminated and no pigment is produced. The unusual property of this receptor element is that it can no longer be excised in response to the mutator product. Thus in such a_2^{m-1} kernels, pigment is produced only in sectors in which Spm is missing or inactive; when two Spm are present, pigment is produced only when both are inactive. Since two inactivation events are less probable than one, a_2^{m-1} kernels with two Spms have fewer pigmented spots than those with one Spm.

With kernels containing the special a_2^{m-1} allele together with an active Spm, and one that is usually inactive for a longer period of time, one would expect to see a pattern of pigmented spots characteristic of a single, active Spm. But, surprisingly, kernels with a double dose phenotype are obtained⁵⁵, reflecting

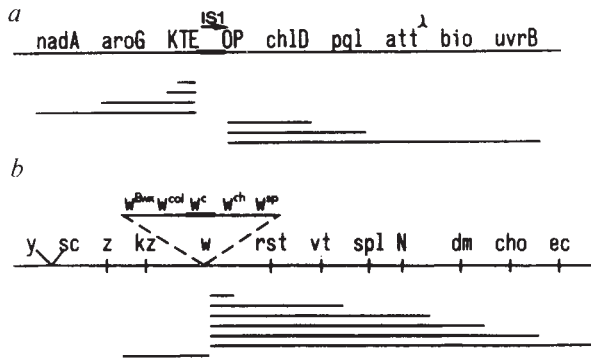


Fig. 6 a, Deletions induced by *IS1* in the galactose operon of *E. coli* and (b) by the controlling element in the white locus of *D. melanogaster*. Data taken from refs. 47 and 64. Horizontal bars indicate extent of material removed in various classes of deletion derivatives.

two active Spms. The originally inactive element is not turned on permanently in this process, however, and ceases to be expressed when its active counterpart is removed by meiotic segregation.

The observation of trans-activation implies a number of features of Spm, which have been incorporated in the model shown in Fig. 5(c). First, the temporary nature of the trans-activation suggests that it does not involve structural alteration of the inactive element. It follows that the S gene and a promoter for the S gene represented by P2 must be arranged in potentially transcribable orientation within the inactive element. The inactive condition of this element can then be accounted for by assuming that P2 is inactive unless stimulated by activator product, supplied in this case by an active Spm. For normal expression of Spm, a second promoter, P1 is proposed, from which transcription occurs spontaneously, leading to expression of the A gene. In addition, the double dose phenotype observed during transactivation indicates that each element is inactivated independently to produce pigmented spots. From this observation it can also be deduced that both the A gene and the S gene are situated downstream from P1 and P2, and that expression of the A gene triggers an autocatalytic cycle of expression of Spm.

Chromosomal rearrangements involving controlling elements

In the preceding sections we have summarised how the integration, excision, and transposition of elements of the Spm system and the action of certain Spm products function to control the expression of some loci in maize. But controlling elements, like IS sequences, have also been shown to enhance the incidence of inversions, deletions, and duplications of adjacent chromosomal regions, although much less attention has been devoted to this phenomenon. In maize, rearrangements of this kind have been observed in particular with the element dissociation⁴. There is evidence that elements resembling IS elements also occur in *Drosophila*^{61,62}, where they are frequently associated with chromosomal rearrangements. Detailed analysis of such processes has been facilitated by the many well-mapped genetic markers of this organism and by the banding patterns of its salivary gland chromosomes which permit visual detection of structural alterations. In one case, a transposable element seems to have been involved in a duplication of part of the white locus for eye colour⁶³. An even more striking example is that of deletions found in association with another controlling element in the white locus of white-crimson mutants (Fig. 6b); in direct analogy to *IS1* mediated deletion in bacteria, these deletions all start at the white locus and extend in either direction, ending at non-random points in the chromosome, resulting in distinct classes of deletion⁶⁴. In this process the controlling element, like *IS1*, is retained in the deletion derivative where it can cause a second round of deletion⁶¹.

The prevalence of IS sequences and controlling elements in organisms as diverse as *E. coli*, *Zea mays* and *Drosophila* suggests that they may be of general biological significance. There are, moreover, hundreds of other organisms that possess 'mutable genes', and exhibit phenotypes similar to those caused by controlling elements (see ref. 9). Furthermore, we have already noted that IS-sequences are natural residents of the wild-type *E. coli* chromosome, and there is evidence that controlling elements also exist at unknown positions of wild-type maize chromosomes, from which they can be 'dislodged' by irradiation⁶⁵, or mechanical disruption of the chromosome^{4,5,66}. Whether they exert control functions at these positions or are simply kept in reserve as prefabricated units for the evolution of new control circuits remains unclear.

The large scale rearrangements found in association with such elements provide interesting grounds for speculation. Duplication, for example, is regarded by many as an indispensable process in evolution, producing a copy of genetic information with which mutational experiments can be conducted at lower risk and exempt from immediate selective pressure^{67,68}. Inversions, deletions, and transpositions, on the other hand, may serve to create new nucleotide sequences or even new genes at fusion points. These processes may also represent important evolutionary means for reshuffling genetic information, uncoupling genes from old regulatory signals and linking them to new ones. This would provide adaptive flexibility while permitting conservation of vital or successfully evolved genetic information. Sequences analogous to IS sequences or their termini may serve as substrates for recombinational events leading to such rearrangements.

One recent observation suggests that chromosomal rearrangements may also play a part in differentiation. The variable and constant parts of the immunoglobulin light chain seem to be coded by two different genes that are fused and expressed as a joint polypeptide⁶⁹. In embryonic cells these genes seem to be separated, since they are located on different DNA restriction fragments. But in certain myeloma cells, which are thought to represent advanced developmental stages, arrested at a certain point, the two genes are found on the same DNA fragment. This has been interpreted to mean that fusion of these genes occurs curing development through deletion of the intermittent material, inversion, or transposition.

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articles

Protein–DNA and protein–hormone interactions in prealbumin: a model of the thyroid hormone nuclear receptor?

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High resolution X-ray analysis of the hormone-binding protein prealbumin has shown that it has a structural complementarity to double-helical DNA. The proposed binding site is composed of two symmetry-related β -sheets containing a pair of helically disposed arms, which can interact with the bases in the wide groove of DNA. A palindromic target sequence is indicated by the symmetry of the protein. The two identical thyroid hormone binding sites on prealbumin are located in a channel that runs completely through the molecule. These two structural features suggest prealbumin as a model for the thyroid hormone nuclear receptor, providing a number of detailed predictions of its properties.

THE generally accepted model for the expression of hormone activity gives a central role to the hormone receptor. Receptors are presumed to be proteins that have two essential properties: the ability to form extremely tight and specific complexes with particular hormones, and the ability to convert the interaction into a signal that initiates the response of the target cell. Although the receptors for many hormones are located in the outer membrane of the target cell, there is increasing evidence that at least one class of receptor for the thyroid hormones is located in the cell nucleus^{1–4}. Since these hormones exert their effects primarily on gene expression, it seems probable that their receptors may be able to initiate this response through binding to the double-helical DNA in chromatin, but little is known of their mode of action at the molecular level.

The thyroid hormones are water-insoluble molecules that require specific binding proteins in the plasma and the cell

cytosol to enable them to gain access to the nuclear receptors. In an attempt to define the nature of the interactions between the thyroid hormones and their binding proteins, we have been working on the X-ray structure of prealbumin, one of the two thyroxine-binding proteins in human plasma. Prealbumin has a molecular weight of 55,000 and is composed of four identical subunits of 127 amino acid residues⁵, arranged with tetrahedral symmetry⁶. The protein has two hormone binding sites whose association constants⁹ for L-thyroxine (T_4) are $1.05 \times 10^8 \text{ M}^{-1}$ and $9.55 \times 10^8 \text{ M}^{-1}$; triiodo-L-thyronine also binds at these sites about an order of magnitude less strongly. The X-ray results indicate that these two sites are structurally identical and are deeply buried in a narrow cylindrical channel that runs through the centre of the protein molecule. Prealbumin also carries independent binding sites for retinol-binding protein (RBP) (ref. 10), the specific plasma carrier of vitamin A. There are apparently four equivalent binding sites for RBP (ref. 11), but information on their location and interactions is at present lacking.

We have now discovered, however, that part of the surface of the prealbumin molecule is complementary in size and shape to the stable B-form of double-helical DNA. This putative DNA-binding site, which has the form of a deep semi-cylindrical groove equipped with a pair of helically disposed arms, is constructed almost entirely from a symmetry-related pair of β -sheets. Although in principle the great diversity of protein structure allows binding sites for any ligand to be constructed, no detailed model of an interaction between native, double helical DNA and a globular protein has yet been proposed. The site we observe is almost entirely composed of regular protein structure, and therefore provides a general model for protein–DNA interactions, especially as the mode of binding that is

indicated incorporates many of the features that have been proposed for such interactions¹².

Because prealbumin has no known function that may involve DNA binding, nor as a soluble plasma protein is there any compelling reason to anticipate such a function, the significance of the structural complementarity to DNA is not immediately obvious. In particular, we cannot rule out the possibility that the site is merely a coincidental feature of the molecular structure, without biological significance. But the closeness of fit and the mode of interaction with the standard DNA double helix suggest that we should seriously consider the alternative possibilities that either prealbumin has so far undetected DNA-binding function, or that it is related to a protein that does have such a function. Of these possibilities the first can be tested either by careful model building of the proposed prealbumin-DNA complex or by direct binding studies, while the second can only be approached at present by speculative arguments. Nevertheless we feel justified in considering the second possibility in more detail because, as the one known protein whose function combines both thyroid hormone and DNA binding, the thyroid hormone nuclear receptor is the most obvious potential relative of prealbumin, implying that the interactions we observe in prealbumin may shed some light on the properties of the receptor itself about which little is otherwise known. These considerations lead us to interpret the presence of a DNA binding site on prealbumin as indicating the possibility that some or all of the thyroid hormone binding proteins belong to a family whose members have evolved from a common ancestor, and in particular that prealbumin and the receptor have evolved from an ancestral protein whose structure encompassed both hormone and DNA binding sites. Evidence for a relationship between prealbumin and the other known thyroid hormone binding proteins (the receptor and the plasma protein, thyroxine-binding globulin, TBG) is limited by our lack of knowledge of the characteristics of these proteins, especially of their amino acid sequence. The only known properties of the receptor, its molecular weight¹³ and the general chemistry of its hormone binding site¹⁴, are similar to prealbumin; while prealbumin and TBG share the same molecular weight, subunit structure, single strong T_4 binding site per tetramer, and the same extreme stability towards denaturants¹⁵. Although this evidence is circumstantial and meagre, it does lend some support to the proposal. We therefore present the details of the protein-hormone and protein-DNA interactions in prealbumin for consideration as a model of the thyroid hormone nuclear receptor. The model predicts a number of verifiable properties of the receptor, including its molecular properties, the nature of its

hormone and DNA binding sites, and a possible pathway of interaction between them.

X-ray analysis

A preliminary description of the tertiary and quaternary structure of the prealbumin molecule determined from a 2.5-Å resolution Fourier map phased with three isomorphous derivatives has been reported¹⁶. Following this preliminary analysis the complete amino acid sequence of prealbumin has been determined⁸, and readily incorporated into the X-ray model. This model has now been partially refined against 1.8-Å resolution X-ray data—the practical limit of the diffraction pattern—to give an overall *R* factor of 29% for the 24,000 unique reflections, where

$$R = \frac{\sum ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum |F_{\text{obs}}|} \times 100$$

Although the refinement is being continued, the present stage enables us to give a reliable picture of the molecule and its binding sites.

The prealbumin monomer is a classic 'β-barrel' molecule, with eight β strands organised into two four-stranded sheets. The whole molecule is a tetramer with subunits arranged tetrahedrally. The β sheets represent the major points of subunit interaction: monomers associate into dimers by antiparallel hydrogen bond interactions between equivalent sheets giving the dimers two eight-stranded sheets; the dimers associate in the whole molecule by opposing equivalent eight-stranded sheets face-to-face in the centre of the molecule. The prealbumin molecule shown in Fig. 1 is therefore a very simple structure composed of four eight-stranded β sheets—an 'inner' pair sandwiched between an 'outer' pair. A remarkable feature of the structure is that the inner sheets are not in contact but form, or are separated by, an open channel that runs through the molecule.

Low resolution X-ray analysis¹¹ of prealbumin complexed with its hormone ligands, T_3 and T_4 , has shown that they are located at two symmetry equivalent sites located deeply within the central channel. As with some other proteins, it has proved difficult with prealbumin to define conditions in which the ligands will bind reliably in the crystals. We suspect the reason for this is the very low solubility of the hormones in the mother liquor of crystallisation, but whatever the reason, we have so far been unable to collect the high resolution three-dimensional X-ray data that is needed fully to define the nature of the

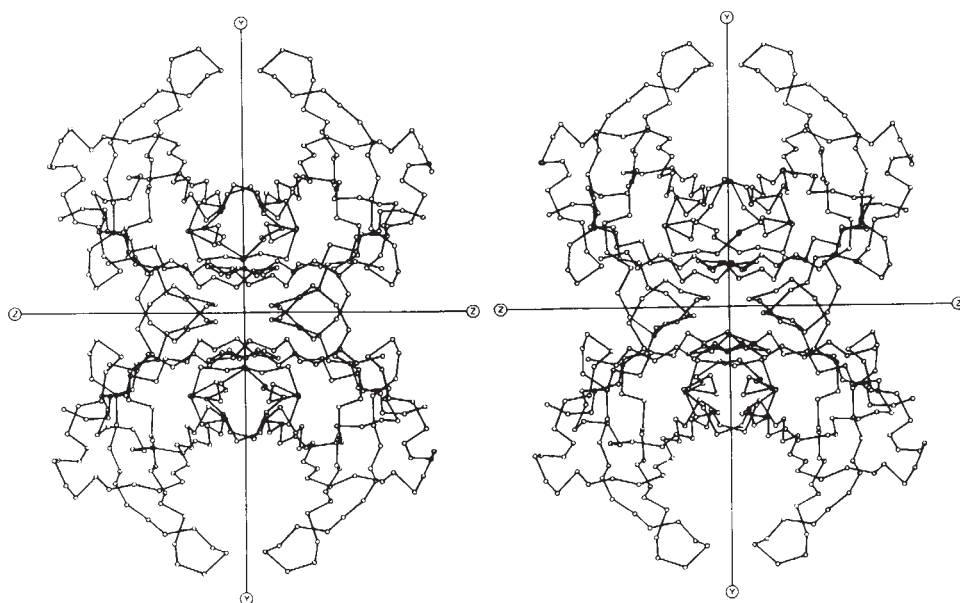
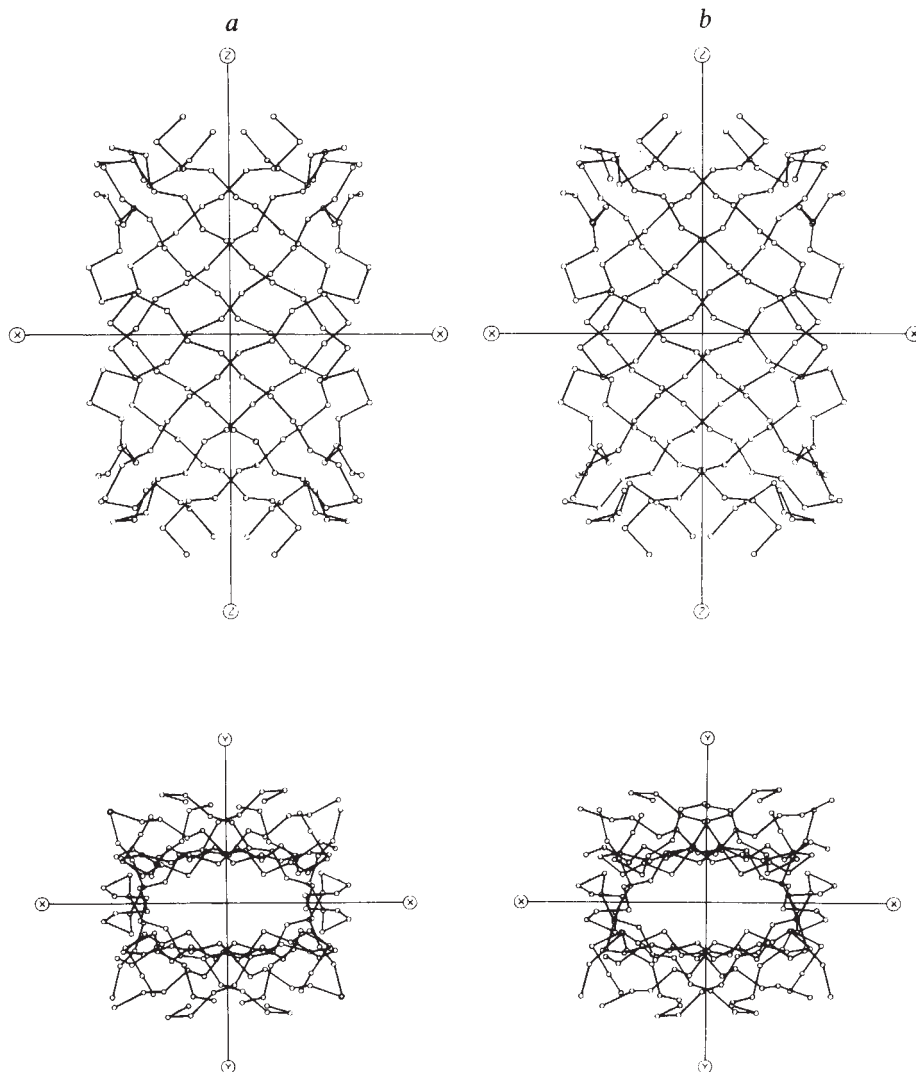


Fig. 1 A stereo drawing of the α -carbon positions of the prealbumin tetramer looking down the molecular *x* axis. The hormone binding sites are located in the channel that runs horizontally through the molecule parallel to the *z* axis. The proposed DNA binding sites are located at the top and bottom of the figure, so that the DNA is located with its helix axis parallel to *x*.

Fig. 2 Stereo diagrams showing the organisation of the polypeptide chains of the four subunits around the central channel; *a*, looking down the *y* axis; *b*, looking down the *z* axis. The circles represent carbon positions.



protein interactions. However, we have been able to collect high resolution data in projection that seem to give results fully consistent with the previous low resolution three-dimensional maps.

The thyroxine binding site

The central channel that contains the thyroxine binding sites can be seen in Fig. 1 running horizontally through the centre of the molecule. Stereo views of the local structure of the channel are shown in Fig. 2, looking down the *y* axis and the *z* axis (the axis of the channel). The surface of the channel is tightly organised from the polypeptide chains of the inner, four stranded β sheet of each of the four subunits, and takes the form of a 16-stranded β cylinder about 50 Å long. The molecular 222 symmetry divides the channel into two symmetry-related halves clearly showing that the two binding sites are identical. In addition one of the molecular twofold axes coincides with the channel axis showing that each site itself has twofold symmetry. Figure 3 shows one half of the channel with the side chains that project into it. The chemistry of the channel is characterised by three elements arranged linearly along it, which reading from the centre are: (1) a hydrophilic patch formed by the hydroxyls of the Ser 117 and Thr 119 pairs of residues; (2) a hydrophobic patch formed by the methyl groups of the Leu 17, Thr 106, Ala 108, Leu 110, Thr 119 and Val 121 pairs; and (3) a group of charged residues including the paired side-chains of Lys 15, Glu 54 and His 56. The absence of any of the molecule's 48 hydrophobic aromatic residues from the channel is both clear and striking. An analysis of the free space around the axis of the channel was carried out by calculating van der Waals

cross sections in 1-Å thick sections along its length. The analysis showed that there is a cylindrical hole along its length with a fairly constant diameter of about 8 Å, except at two symmetry-related positions some 4 Å on either side of the centre of the channel where the close approach of the paired side chains of Leu 110, Ser 115 and Ser 117 cause a marked local constriction.

The low resolution X-ray analysis of complexes of prealbumin with both T_3 and T_4 , showed that the hormones are bound exclusively at two symmetry-related sites buried deeply within the central channel (see Fig. 8 of ref. 16). The difference electron-density envelopes for both ligands were elongated along the axis of the channel and extended from about 4 Å from the centre to about 16 Å. Interpretation of these features suggest that the two hormones are bound in much the same way, located between the constriction near the Ser 117-Thr 119 pairs of side chains and the Lys 15-Glu 54 pairs of charged residues, with their long axes more or less parallel with the axis of the channel. The identity of the two binding sites by symmetry confirms the proposal^{9,17} that the difference in binding constants implies negative co-operativity between the sites. The constriction observed near the centre of the channel would prevent access from one binding site to the other, indicating that the interactions are of the site-site rather than ligand-ligand type (compare refs 9 and 17).

The 3-Å resolution projection maps of the ligands have been used to attempt to locate the iodine substituents of the hormones within the overall electron-density envelopes observed in the low resolution maps. Although fully self-consistent results have been obtained, projection maps of proteins can be very misleading and the interpretations given below must be regarded

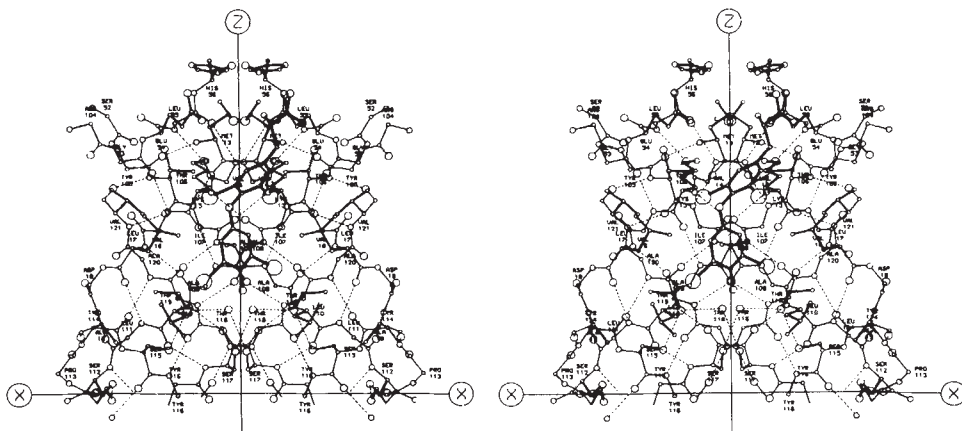
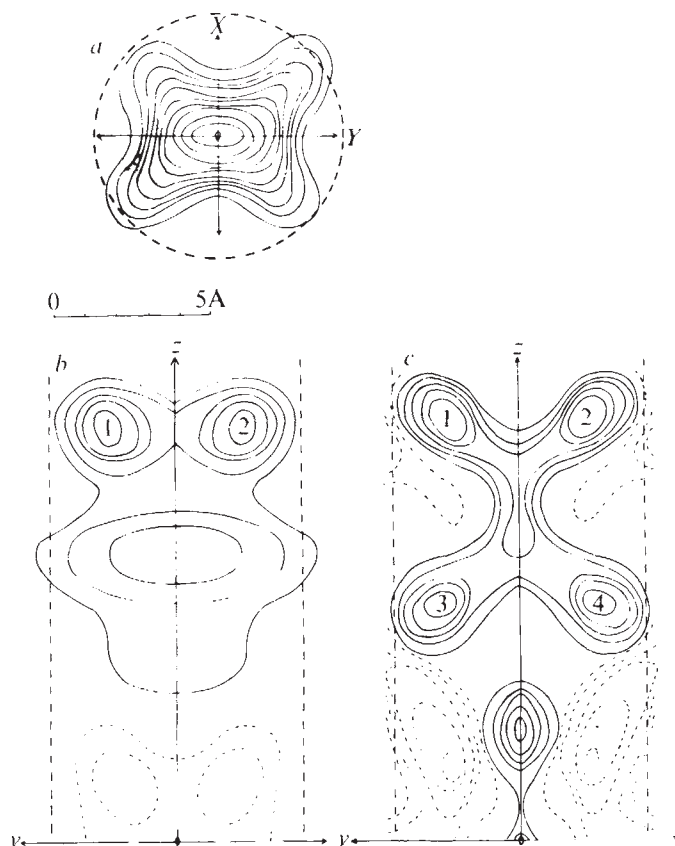


Fig. 3 Stereo diagram of the atomic arrangement in a single hormone binding site. This drawing corresponds to the upper half of Fig. 2(a). The main chain is shown in light line and side chains that point into the site are shown in medium line. The position of the T_4 molecule, shown in heavy line, was derived by fitting the coordinates of Cody¹⁴ to the iodine positions indicated in the projection maps.

as provisional until three-dimensional high resolution data on ligand binding is obtained. The difference map of T_3 binding projected down the z axis (the axis of the channel and approximately the long axis of the hormone) is shown in Fig. 4a. It can be interpreted as indicating that the benzenoid rings of the hormone are roughly at right angles as expected (the bulky iodine substituents cause other orientations to be energetically unfavourable), and are orientated at about 45° to the molecular x and y axes. The equivalent maps of T_3 and T_4 projected down the x axis shown in Figs 4b and c contain discrete peaks consistent with Fig. 4a, that can be interpreted as representing the various iodine substituents of the hormones. It can be seen that the pairs of peaks labelled 1 and 2 further from the centre of the channel are coincident in both maps, but that the inner pair labelled 3 and 4 in the T_4 map is replaced by a diffuse

Fig. 4 3-Å resolution difference maps of the binding of (a) T_3 projected down the z axis; (b) and (c) T_3 and T_4 respectively, projected down the x axis. Contours are at equal but arbitrary levels; positive in full line, negative in broken line. The confines of the channel, assuming a constant diameter of 8 Å, are shown in heavy broken line.



region of density in T_3 . The simplest interpretation of the two maps is that the common pair of peaks represent the 3,5 pair of iodines in each hormone, that the inner pair of peaks in T_4 represent its 3',5' pair of iodines, and that the diffuse density in T_3 represents alternative positions of its single 3'-iodine.

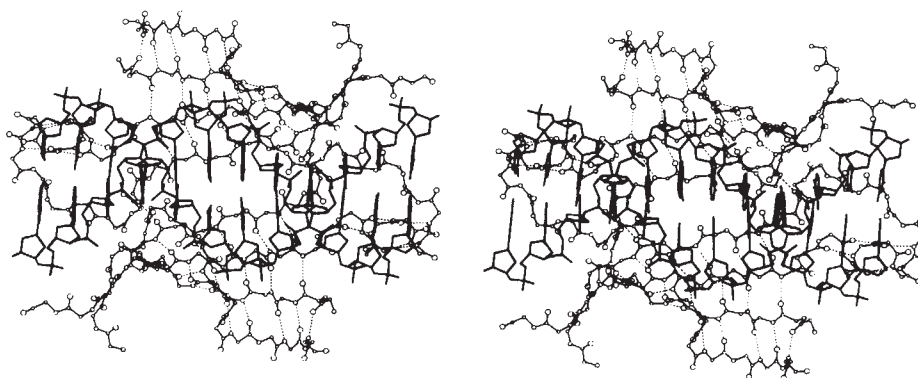
Taking the interpretations of the projection maps together it is possible to define a tetrahedron of iodine substituents for T_4 that is generally consistent in size and geometry with that found in the structures of the crystalline hormone^{18,19}. However, to obtain the points of interaction between the iodines and the protein, an ambiguity concerning the orientation of the tetrahedron—are the peaks 1 and 4 of Fig. 4c above the plane of the projection and peaks 2 and 3 below, or vice versa—has to be resolved. A provisional solution has been achieved by finding that one orientation can be fitted in the channel without producing unacceptably close contacts with the protein, but that the other cannot. Assuming the former orientation to be correct (shown in Fig. 3) each pair of iodines can make favourable hydrophobic contacts with protein side-chains. Each of the 3',5'-iodines lies between and in contact with the side chains of Leu 17 and Leu 110, and each of the 3, 5-iodines fits into a pocket lined with the methyl groups of Thr 106, Ala 108 and Val 121 and the β - and γ -methylenes of Lys 15. These positions are also consistent with the interaction of the hormone's 4'-hydroxyl, through a well defined water molecule, with the hydroxyls of the Ser 117 and Thr 119 pairs of residues in the hydrophilic patch, and with the interaction of the hormone's α -carboxylate and α -amino group with Lys 15 and Glu 54 respectively. This latter interaction, and with it the broad outline of this proposal for hormone binding, is strongly supported by affinity labelling experiments on prealbumin using dansyl chloride²⁰ (a competitive inhibitor of T_4) and bromoacetyl- T_4 (ref. 21), both of which have been found covalently attached to Lys 15.

The DNA binding site

Our attention was drawn to the possibility of a DNA-binding site on prealbumin by the remarkable cylindrical-helical depression in the outer surface of the molecule, shown in Fig. 1. This site is formed by the 'outer' β sheets of two symmetry-related subunits. The normal right-handed twist of the sheets coupled with the symmetry induced by the molecular twofold axis parallel to y gives rise naturally to a concave cylindrical structure, that is given its helical character by the extended hairpin loops made by the two outer strands of each sheet. The structure so formed has a length of about 40 Å, a diameter of about 25 Å and a helical pitch of about 33 Å.

The extent to which this site is complementary to double-helical DNA was explored by model building, using an interactive computer display system²². The coordinates used were those of the main-chain atoms of prealbumin from the latest stage of refinement, and a standard set for all the atoms of the B form of the DNA double helix²³. These coordinates were maintained throughout the fitting, which therefore corresponded

Fig. 5 Stereo diagram of the proposed prealbumin-DNA complex looking down the y axis, with the x and z axes horizontal and vertical respectively. The main chain atoms of the outer β -sheet are shown in light line with the hydrogen bonds in broken line. The DNA is drawn in heavy line. All the atoms in the diagram are related by the twofold axis parallel to y that is positioned at the centre of the diagram.



to the mating of two rigid molecules. The first step in locating the DNA in the site was carried out by making the molecular y axis of the protein coincide with one of the twofold axes of the DNA helix that runs between each base pair perpendicular to the helix axis. When the protein and DNA twofold symmetry axes coincide, only two degrees of freedom remain: rotation of the DNA around the axis relative to the protein, and translation of the DNA along the axis relative to the protein. Rotation of the DNA around the axis was seriously restricted by the helical arms to a narrow range of orientations in which the DNA helix runs closely parallel to the axis of the cylindrical channel. In this orientation the helical arms of the protein fit closely into the wide groove of the DNA double helix. A rotation of 180° from this orientation—the only alternative position—is not as favourable because the helical arms are too large to fit into the narrow groove of the DNA. Translational adjustment of the DNA along the common symmetry axis was relatively easy because it corresponds to bedding the DNA down the channel, making due allowance for the protein side chains.

The final fit of the DNA to prealbumin obtained from the computer display is shown in Figs 5 and 6. Its characteristics are as follows.

- (1) The twofold axis of symmetry of the protein coincides with the twofold axis of symmetry in the DNA.
- (2) The DNA double helix lies in the pronounced cylindrical depression in the protein molecule with its helix axis parallel to the axis of the depression.
- (3) The pair of helical arms of the protein fit closely into the wide groove of the DNA double helix. This is the most striking feature of the fit because it could allow the arms to interact with the DNA bases whose edges are exposed in the wide groove. We therefore consider that the helical arms can act as 'reading heads' that probe the DNA for a compatible base sequence. The twofold symmetry relation of the arms would necessarily imply that such compatible base sequences must be palindromic. But Fig. 5 shows that this is not necessarily true for the two base pairs at the very centre of the binding site which are in fact more distant from the protein surface than the base pairs on either side. This relieves the requirement for an exact palindrome at the centre of the interaction site as has been observed for the *lac* operator²⁴ and the *Haemophilus* endonuclease specificity site²⁰.

We have not yet carried out the careful model building of DNA in the proposed site on prealbumin that might tell us whether the protein, in addition to having an appropriate structure, also has the appropriate side chain distribution for favourable interaction with DNA. At present, therefore, we can only note that the proposed binding site on the protein is especially rich in ionic side chains; ten lysines, four histidines, eight glutamates and six aspartates are located in positions where they could interact with some part of the DNA. In addition Trp 41 is located in a totally exposed position on the helical arms with its indole ring parallel to and between neighbouring base pairs, suggesting that intercalation should be considered in any possible scheme of interaction.

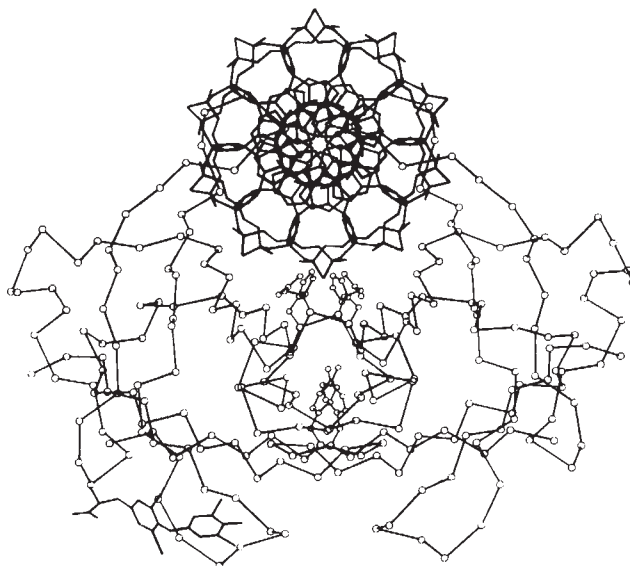
Finally, any model of a hormone nuclear receptor should

indicate the possibility of a means of communication between the hormone and DNA-binding sites. That some structural response to thyroxine binding takes place in the prealbumin molecule is indicated by the negative cooperativity of binding. The nature of this structural response, which may alter the geometry and properties of the DNA-binding site, can be determined by high resolution X-ray studies of hormone binding. In the absence of these studies we can only note that the separation and orientation of the symmetry-related pairs of β -sheets that form the DNA site, could be controlled by Tyr 116 (whose position is shown in Fig. 6) whose hydroxyl is inserted between the inner strands of the two β -sheets, forming a hydrogen-bond bridge between them. The residue following this tyrosine, Ser 117, is located in the hormone binding site and is hydrogen bonded through a water molecule to the 4'-OH of thyroxine. Although there may be other possibilities, Tyr 116 is the most obvious means of communication between the sites, because it is uniquely situated with its peptide involved in the β -structure of the hormone binding site and its hydroxyl involved in the hydrogen bonding network of the β -sheets that form the DNA binding site.

Conclusions and predictions

The most important general conclusion from this work is that the typical binding site depression of a globular protein molecule

Fig. 6 A drawing of the proposed prealbumin-DNA complex looking down the x axis, with the y and z axes vertical and horizontal respectively (compare with Fig. 1). The main chain of the prealbumin dimer is shown in light line; the circles represent the α -carbon positions. The symmetry-related pair of tyrosines 116 are shown at the lower centre, and two pairs of side chains that point into the DNA binding site are shown in the centre. The DNA double helix is seen end-on in heavy line at the top, and one T₄ molecule in its (half) site is drawn in heavy line at the lower left.



can take on the helical character necessary for combination with native DNA when the binding region is composed of a symmetry-related pair of β -sheets. If the outer strands of the sheets 'peel off' from the body of the protein, as occurs in prealbumin and also, in different contexts, in a number of other β -structured proteins, a pair of helically-disposed 'arms' are formed that can fit into the grooves of the DNA double helix where they can probe the nucleotide bases for a complementary sequence. As a consequence of the twofold symmetry of the site, the complementary base sequence must necessarily correspond either to an exact palindrome or alternatively to a palindrome with a gap at its centre. The latter case arises because the arms fit the outer parts of the target sequence more closely than its centre. Although the particular structure of prealbumin suggests a potential interaction with a six or eight base palindrome, it can easily be extended to provide a general basis for protein-DNA interaction, that can account for the four-base palindromic target sequences of some restriction enzymes²⁶⁻²⁸ and the 20-base 'gapped' palindrome of the *lac* operator²⁴. Details of this general model will be published elsewhere.

In prealbumin the thyroxine binding sites are located deeply inside a cylindrical channel that runs completely through the molecule. When bound at these sites the hormone is almost completely removed from the surrounding medium. The binding sites have twofold axial symmetry to which the hormone is surprisingly well-suited and provide individually favourable microenvironments for each of the characteristic substituents of the hormone. Minor side-chain substitutions in a site of this kind, such as possibly the inclusion of aromatic residues at the iodine binding sites, could readily account for the very tight binding exhibited by receptors.

Finally we consider the possibility that the presence of a potential DNA-binding site, in addition to its thyroxine site, makes prealbumin a useful model of the thyroid hormone nuclear receptor. This possibility can be tested by verifying or contradicting the following predictions on the properties of the receptor that can be derived from the model: (1) it is a tetramer; (2) it is unusually stable and not easily dissociated into subunits; (3) its polypeptide chain is largely organised into β -structure; (4) it has two hormone binding sites; (5) it interacts with a segment of DNA 10-12 base pairs in length; (6) this segment

contains a palindromic sequence of six to eight bases. Two other properties of the receptor, its molecular weight¹³ and the linear arrangement of hydrophilic, hydrophobic and ionic groups in its hormone binding site¹⁴, are not included in these predictions because they have already been determined, and are in agreement with the model.

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Separation of membrane currents using a *Paramecium* mutant

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The net membrane current of the electrically excitable membrane of Paramecium during step depolarisations was measured using voltage-clamp techniques. The mutant, pawn B, lacks a functional Ca channel. Thus the difference between the total current measured in the wild type and the leakage and rectification currents measured in the pawn mutant is the Ca current. These findings were confirmed by ion substitution experiments. The possibility that inactivation may be apparent only, and caused by a hypothetical, superposed, Ca-induced, rectifying K⁺ current was eliminated.

IN an attempt to study the molecular biology of excitable membranes in ways not possible in nerve, behavioural mutants with defective membrane functions have been isolated in *Paramecium*. *Paramecium* is a unicellular animal which swims by beating its cilia. The direction of its ciliary beat is controlled by the electrically excitable membrane^{1,2}. Extensive electrophysiological studies produced evidence of the existence of Ca and K channels in the *Paramecium* membrane³⁻⁸. One of the difficulties of membrane-physiological investigations in general is that only the net trans-membrane current or the voltage change due to this current is measured by the electrode. Analysis of the properties of

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a single kind of channel requires a separation of different ion currents. Traditionally, this is done with drugs or by ion substitution^{10,11}. The same purpose can be accomplished by genetic means. We separate the inward Ca^{2+} current by comparing the total current in wild-type *Paramecium* and that in a mutant which lacks a functional Ca Channel^{4,9}. To measure the total membrane current, we use constant voltage pulses during step depolarisations. We find that the Ca current inactivates in time, similar to the Na currents described in nerve¹² but different from Ca^{2+} currents described in barnacle muscle¹³ and at the squid giant synapse¹⁴. This finding is supported by ion substitution experiments done on single wild-type cells. Suspensions are removed that the apparent time course of the Ca^{2+} current may be partially masked by a Ca-induced K^+ rectifying current.

Wild type compared with the mutant 'pawn'

When wild-type paramecia hit a physical obstacle or enter an undesirable ionic environment, they avoid it by swimming backwards for a short distance and then swimming forwards again in a different direction¹⁵. The electrical excitability of the membrane controls backward swimming. A small depolarisation results in a graded action potential whose inward current is carried by Ca^{2+} (refs 3, 5). The increase in internal Ca^{2+} concentration then causes the cilia to reverse the direction of their power stroke (ciliary reversal) by a yet unknown mechanism¹⁶.

Named after the chess piece, 'pawns' are mutants which cannot swim backward^{17,18}. Electrophysiological studies traced the defect of these mutants to the malfunction of the Ca channels^{4,9}.

Paramecium tetraurelia, previously known as *P. aurelia*, species 4 (ref. 19) was used in these experiments. The wild type was stock 51S and the pawn mutant was stock d4-95, genotype *pwB pwB* (refs 4, 17). Voltage was clamped using standard techniques²⁰. Cells, in a buffer solution containing 1 mM CaCl_2 , 1 mM HEPES (Sigma), 0.5 mM KOH, 3.5 mM KCl, 10^{-7} M EDTA, pH 7.0, were impaled with micro-electrodes filled with 3 M potassium acetate and having a resistance of 10–15 M Ω . The potential was held at resting level and stimulus pulses were given at a frequency of about 1 Hz. The voltage stabilised at the command level 500 to 1,000 μs after a step change in the command. At the end of an experiment the voltage-measuring electrode was withdrawn to measure the reference potential. Activation of the Ca^{2+} inward current is fast, so that the beginning of activation could not clearly be seen, even in the best preparations. It is technically difficult to get stable preparations of *P. tetraurelia* with the low resistance electrodes which are required if the potential is to be changed quickly enough to see inward currents clearly.

Sample traces are shown in Fig. 1. The current traces from wild-type cells (Fig. 1a, c) show a transient inward current lasting about 5 ms and then a steady-state outward current in response to a step depolarisation. A step depolarisation of pawn cells results in a steady-state outward current but there is no detectable inward current (Fig. 1b) even when viewed at high gain (Fig. 1d). Steady-state outward currents of pawn and wild type are not detectably different over the range of voltages studied (Fig. 2), and the voltage dependence of both peak and steady-state currents is similar to that in *P. caudatum*²¹. Depolarisations to nearly 0 mV from the resting potential of ~ -23 mV result in a transient inward current and a steady-state outward current whose magnitude is about equal to that of the leakage current. Steady-state outward currents increase steeply as a function of voltage for step depolarisation above 0 mV. As in other excitable membranes, this voltage-dependent outward current is called the delayed-rectification current, and it is probably due to the opening of K channels. At large depolarisations the outward current activates so rapidly that the inward current cannot be seen clearly.

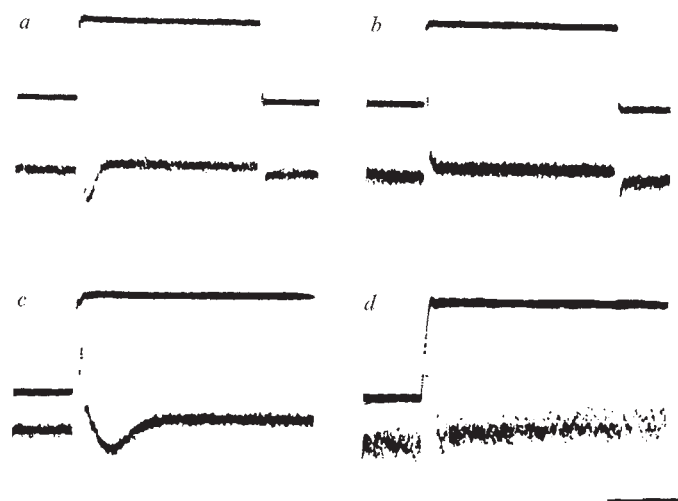
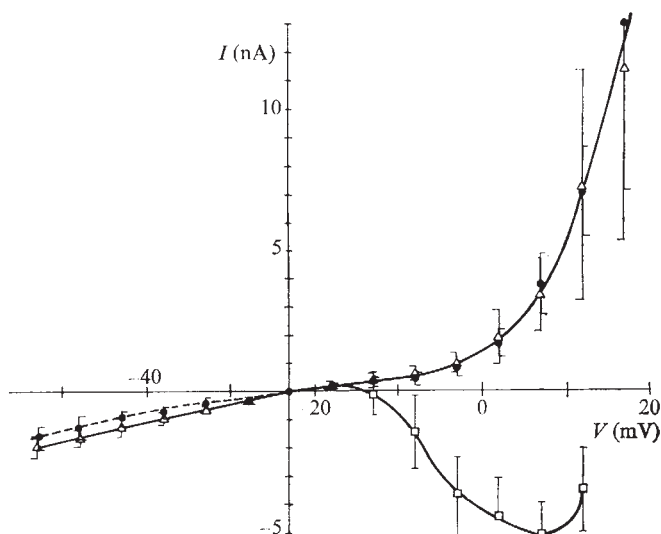


Fig. 1 Sample traces of voltage-clamp experiments in two strains of *Paramecium tetraurelia*. a, c, Records from wild type; b, d, records from the mutant 'pawn' cells. In each case the upper trace shows the voltage and the lower trace shows the associated current. In (a), a transient inward current is followed by a steady-state outward current. Record (c), from a different cell, shows a similar event at greater sweep speed. Record (b), and (d) show that pawns lack the transient inward current; (d) is recorded at higher gain and faster sweep than (b). Calibration: a and b, 20 mV, 10 nA, 10 ms, seven superimposed sweeps; c, 20 mV, 10 nA, 4 ms, 10 superimposed sweeps; d, 20 mV, 5 nA, 4 ms, 10 superimposed sweeps. Holding potentials are 20 mV, 24 mV, 25 mV, and 25 mV for a, b, c, and d, respectively.

Pawn lacks a transient inward current, but there is no measurable difference in the characteristics of the steady outward currents between pawn and wild-type cells. The time courses of the transient inward current alone can be calculated by subtracting leakage and rectification currents as measured in pawn from the total current measured in wild type. Figure 3 shows one such calculation for cases with minimal rectification.

Fig. 2 Voltage-current relationship of peak inward ($\square$) and steady-state outward ($\triangle$) currents of wild type, and steady-state outward ($\bullet$) current of pawn mutant. Steady-state outward currents were measured 25 ms after the beginning of depolarising pulses. Minimum inward currents resulted from hyperpolarising pulses. Data were taken from four wild-type cells with holding potentials 23 ± 5 mV (mean $\pm$ s.d.) and five pawn cells with holding potentials 23 ± 3 mV. Points and bars represent mean current magnitudes $\pm$ s.d. Where the slope of the curve is greater, standard deviations are larger. There is no significant difference in the steady-state outward currents between the two stains.



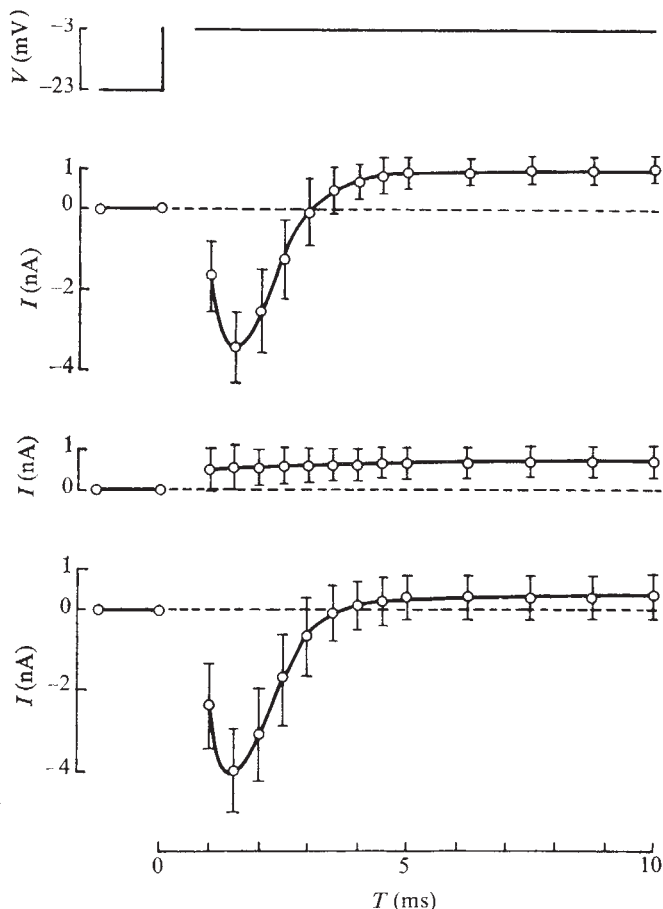


Fig. 3 Average time course of currents associated with step depolarisations of 20 mV in wild type (a) and pawn (b). Mean current magnitudes $\pm$ s.d. were measured at various times from the same four wild-type cells and five pawn cells whose voltage-current characteristics are shown in Fig. 2. c shows the difference between current measured in wild type (a) and pawn (b) $\pm$ s.d. of the difference of the means. Measurements were not made in the first 1.0 ms after the step depolarisation because the voltage was not well controlled during this time.

Variation in measurements makes the comparison of single cells unreliable, so average time courses of membrane currents were determined from four wild-type cells (Fig. 3a) and five pawn cells (Fig. 3b) after a 20-mV step depolarisation. The difference of the two time courses (Fig. 3c) shows the time course of the Ca^{2+} current. The inward current in these experiments begins before the voltage has stabilised. Therefore, the early kinetics of activation are obscured. The Ca^{2+} current apparently inactivates.

Inactivation of Ca current

Figures 1 and 3 show that the inward current diminishes in time. Other experiments were done to test whether the Ca^{2+} current inactivates. To obtain a more accurate measure of the Ca^{2+} current from a single cell, a series of experiments was done in which 99% of the CaCl_2 in the external solution was replaced by MgCl_2 . The results of such an experiment are shown in Fig. 4. The records obtained when the cell was bathed in buffer containing 0.01 mM CaCl_2 and 0.99 mM MgCl_2 resemble those of pawn. They lack the transient inward current but steady-state, outward currents remain unaffected. These results confirm our conclusion that the inward current is a Ca^{2+} current and that this current inactivates.

The effects of Ca^{2+} and Mg^{2+} concentrations on the properties of the membrane were studied systematically. Decrease of external Ca^{2+} lowered the membrane resistance. Increase of external Ca^{2+} increased the resistance and

raised the measured activation potential for the inward current. The measured activation potential for the inward current was also raised when Mg^{2+} was added to the standard 1 mM CaCl_2 buffer. These results suggest that surface-potential effects must be considered in studies of *Paramecium*, and that lowering the divalent cation concentration indirectly affects membrane resistance and the channel's apparent activation potential in addition to changing the driving force of Ca^{2+} across the membrane²².

Acidic phospholipids in the paramecium membrane give the membrane a negative surface charge. This must cause a relatively higher concentration of cations near the membrane than in the bath. Changes in the ion composition of the bath, particularly in its divalent cation concentration, will alter the surface potential and ion concentrations in the vicinity of the membrane, and thus the electric field to which the membrane components are subjected may be quite different from that measured at a distance²³. The experimental result that neither the measured activation potential for delayed rectification nor the hyperpolarisation currents are affected if Mg^{2+} is substituted for Ca^{2+} suggests that Mg^{2+} does substitute for Ca^{2+} in its effects on membrane resistance and surface potential but that it does not cross the membrane through the Ca channels^{22,24}.

The rate at which inactivation is removed when the membrane was held at resting potential was studied by stimulations paired at various intervals. A sample trace of such an experiment is shown in Fig. 5. Two depolarising pulses were given, each 5 ms long. An inward current is the response to a first depolarisation but it is lacking after the second. The time course showing the recovery of the inward current is shown in Fig. 5. It takes 80–90 ms for inactivation to be removed after a 5 ms pulse.

Ca-activated K conductance not involved

A Ca-activated potassium conductance has been shown to exist in a number of systems^{25,26} that possess an inward Ca^{2+} current. It therefore seems possible that the observed loss in time of inward current is not caused by inactivation but by an activation by Ca^{2+} of an outward current which eventually cancels the inward current. The experiment shown in Fig. 6 demonstrates that this is not the case. If we

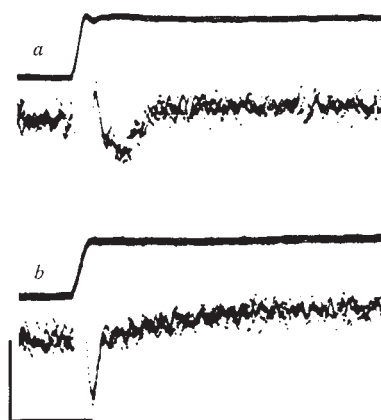


Fig. 4 Separation of currents in a wild-type cell by ion substitution. a, Current response (noisy trace) to a step depolarisation (smooth trace) in a bath containing 1 mM Ca^{2+} . b, Current, response to a similar depolarisation in the same wild-type cell when the bath was changed to one with 0.01 mM Ca^{2+} and 0.99 mM Mg^{2+} . The transient inward current seen in the upper trace is not seen when the external Ca^{2+} concentration is reduced but steady-state currents are equal. This effect is reversible. Due to use of microelectrodes with relatively high resistances, unavoidable when voltage-clamping *P. tetraurelia*, a current oscillation follows the capacitive current at the beginning of the step depolarisation. The membrane potential first was held at the resting potential, -25 mV, then depolarised to -10 mV in each case. Calibration bars: 20 mV, 10 nA and 4 ms. Each record shows four superimposed traces.

suppose that in the steady state there is a Ca-activated K^+ current component which cancels some of the inward current, then a sudden change of potential to more negative than E_K (ref. 21) ($E_K = -38$ mV in *P. tetraurelia*, unpublished observation) would reverse the direction of the K^+ current while the Ca^{2+} current should remain inward. Thus, the Ca^{2+} current and the hypothetical K^+ current which are supposed to cancel at -10 mV should add at a membrane potential below -38 mV. But, the observed hyperpolarising current is the same, with depolarising prepulse and without. There is, therefore, no measurable, long-lasting, Ca-activated K^+ current in the steady state which could be considered responsible for cancelling of an inward current. Ca-activated K channels may well exist in the *Paramecium* membrane (Y. Satow and C. Kung, in preparation). But, these channels seem not to be activated at internal Ca^{2+} concentrations or with kinetics relevant to excitation, in the conditions described in this paper. The relatively slow increase of the inward current during hyperpolarisation is due to activation of a different K conductance which causes an anomalous rectification (Oertel *et al.*, unpublished and ref. 27).

Concentration and conductance of Ca^{2+} per cilium

Two independent studies have shown that the excitation-related Ca channels are located mostly, and perhaps exclusively, in the cilia^{28,29}. Thus it is likely that it is the local concentration of Ca^{2+} in the cilia, and not the concentration in the general cytoplasm, which controls beat direction. An order-of-magnitude estimate can be made of the increase in Ca^{2+} concentration in a cilium during an action potential. For this rough estimate we make the simplifying assumption that the time course of the Ca^{2+} current during an action potential is similar to that measured in voltage clamp conditions. Such an assumption seems reasonable, because the action potentials last so long that almost all channels should be open and inactivated^{3,4}. 8×10^{-17} equivalents of Ca^{2+} are transferred during a 20 mV depolarising pulse. Taking the collective volume of all cilia of one cell to be 1.7×10^{-12} l, the change in Ca concentration in cilia would be about 2.3×10^{-5} M.

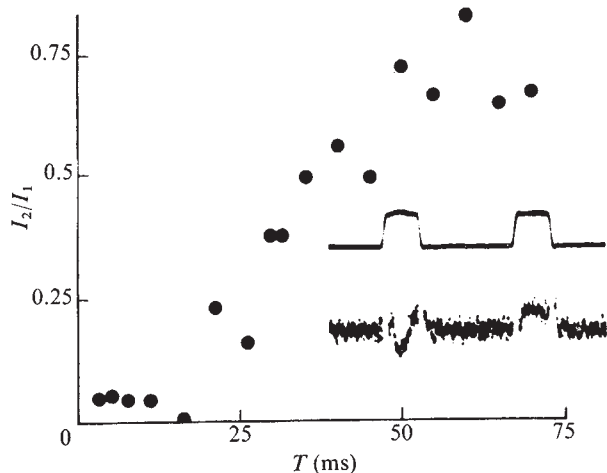


Fig. 5 Time course of return to resting state after inactivation in wild-type paramecium. Two 5-ms depolarisations each of 15 mV were given at various intervals. The ratio of the magnitudes of the second inward current to the first (leakage current subtracted) plotted as a function of time between end of first and second voltage pulse. Inactivation, nearly complete for about 20 ms, was removed after about 80–90 ms. Cell held at -18 mV between pulses. Sample trace is from a different cell with lower resting potential, but shows results more clearly because of smaller current swings after voltage changes.

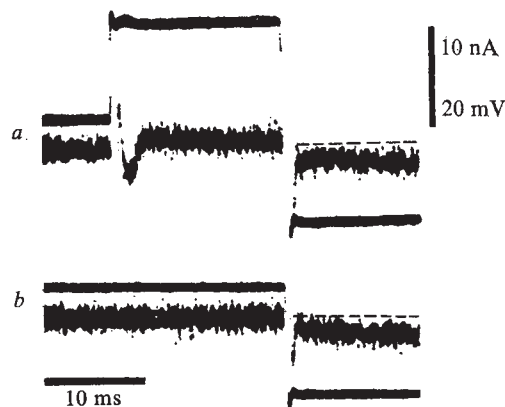


Fig. 6 Transience of inward Ca^{2+} current is not due to slow increase of a masking rectifying current but to an actual decrease of Ca^{2+} current (inactivation). *a*, Current response (noisy trace) to a step depolarisation followed by a hyperpolarisation (smooth trace). *b*, Current response of hyperpolarisation only without a depolarising prepulse. To detect hypothetical currents, assumed to cancel during depolarisation, current responses to hyperpolarisation are compared in *a* and *b*. If transience of the inward current, seen when the cell is depolarised, is due to Ca^{2+} inactivation, the steady-state current should comprise only small rectifying and leakage currents. Hyperpolarisation to a potential (-48 mV) more negative than E_K (-38 mV) must reverse any postulated rectifying currents making all ion currents inward and additive. The observed current following depolarisation in *a*, however, is not detectably larger than in (*b*). Therefore, the inactivation, lasting for 80–90 ms, cannot be explained by postulated long-lasting rectifying currents.

Using paramecia whose membranes were made leaky with a detergent, Naitoh and Kaneko¹⁶ showed that 10^{-6} M Ca is the threshold for ciliary reversal. Our estimate indicates that each action potential can deliver enough Ca^{2+} to cause ciliary reversal. Eckert³⁰ arrived at a similar conclusion by calculating how much Ca^{2+} was needed to charge the membrane capacitance.

It is also possible to make a rough estimate of the Ca^{2+} conductance per cilium. The maximum Ca^{2+} current measured is 4 nA for a depolarisation to 0 mV. If one assumes that the equilibrium potential for Ca^{2+} is about 150 mV, the maximum conductance per cell is 27 nmho, a value in close agreement with that calculated by Schein *et al.*⁹, and the maximum conductance per cilium is only 5 pS.

Discussion

Indirect evidence further supports our finding that Ca channels in *Paramecium* inactivate. Naitoh and Eckert³ and Naitoh, Eckert and Friedman³ showed that the action potential of *Paramecium* is refractory. Since it also shows no undershoot which would indicate a maintained increased permeability to K^+ , the refractoriness of the action potentials must be due to Ca inactivation. Eckert, Naitoh and Machemer² suggest that the *Paramecium* Ca current is at least partly transient. They found that small depolarising voltage-clamp pulses of several seconds produce only transitory reversals and that large and long depolarising pulses produce ciliary reversals which decrease in frequency with time. The transitory reversals observed by these authors last much longer than our calculated Ca^{2+} current. It is, however, difficult to compare time courses of currents and with those of ciliary reversal because little is known about how internal Ca concentration is regulated. Ultimately, it is internal Ca^{2+} concentration, not Ca current, which regulates reversal^{2,16,31}.

The Ca^{2+} action potential in *Paramecium* resembles Ca^{2+} spikes in metazoan systems^{13,14,32–34}. In all cases, the action potentials are graded; Ba^{2+} and TEA^+ , applied externally, change the action potential from graded to all-or-none^{32,33}.

But it is not possible to make generalisations about inactivation. Hagiwara³² and Keynes *et al.*¹³, for instance, report that the Ca^{2+} current in barnacle muscle does not inactivate, whereas Mounier and Vassort³² show that the Ca^{2+} current in crab muscle does inactivate.

We have shown that the *pwB* mutation of *Paramecium tetraurelia* eliminates the transient inward Ca^{2+} current that occurs in the wild type on depolarisation. This observation enables us to separate the Ca^{2+} inward current, much as one uses tetrodotoxin to separate the Na^+ current in nerve cells, from the leakage and rectifying currents.

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letters to nature

Airborne studies of thunderstorm electrification

THERE is considerable controversy^{1,2} surrounding the problem of thunderstorm electrification. Many scientists have maintained that precipitation acquires substantial charges and that field growth to breakdown magnitudes is a consequence of the separation under gravity of the precipitation and the oppositely charged cloud particles. Others have argued that precipitation is incidental to field growth, and have reported lightning from clouds in which the precipitation rates are very low. Of the charging mechanisms involving precipitation, the inductive mechanism^{3,4} has been generally favoured. Under this mechanism rebounding collisions of small cloud particles with the underside of precipitation elements result in the removal from the latter of polarisation charge, and the maximum charge Q_m (pC) that can be acquired by an element of diameter d (mm) in a field E (kV cm⁻¹) is given by

$$Q_m = 5.5Ed^2 \quad (1)$$

We summarise here the results of some airborne experiments conducted in the summer of 1976 in Florida, as part of the Thunderstorm Research International Programme, and subsequently in Socorro, New Mexico. The major objectives of this study were to determine whether substantial currents are carried on precipitation in thunderclouds, and to investigate the relationship between Q , E and d for precipitation particles—and thereby to test the applicability of equation (1) to thunderstorm electrification.

The measuring equipment was carried on the ONR/NMIMT Schweitzer aircraft. This was originally a drone, but it has been modified to accommodate a pilot. Its particular virtue—which renders it excellent for these experiments—is that it flies very slowly (around 40 m s⁻¹). Thus it has a minimal effect on any clouds through which it passes. Its ceiling altitude is 13 km.

The electric field in three mutually perpendicular directions was measured using five appropriately sited field mills. Simultaneous measurements of the charge and size of precipi-

itation particles were made with a novel device mounted under one wing of the aircraft. An aluminium cylinder of length 80 mm and diameter 50 mm was mounted with its axis parallel to the airflow. The charge Q carried on a hydrometeor which passed through the device without touching it was sensed by induction and measured using a charge amplifier. A major problem with such a method is that spurious signals may arise from collisions of hydrometeors with the sensing cylinder. This was circumvented by making the time constant for decay of charge (resulting from such a collision) about 10 times longer than the transit time (and thus the duration of the charge-pulse) associated with the clean passage of a hydrometeor through the cylinder. The two types of pulse could be distinguished unequivocally and only the true ones (clean passages) were accepted. Various practical tests demonstrated that the sensitivity of the charge amplifier was consistent with that derived from an analysis of the circuit. The device was so arranged that it was impossible for hydrometeors to splash into the induction cylinder from any part of the aeroplane. Insulation loss in the charge amplifier—which happened occasionally in very heavy rain—was easily recognised from the recorded signals. Values of Q up to ± 150 pC could be measured to an accuracy of ± 5 pC.

The coincident measurements of the size d of hydrometeors passing through the induction cylinder were effected by means of a shadowgraph technique. A beam of light passed across the cylinder, perpendicular to its axis, and half way along its length. It illuminated a linear array of 64 photo-diodes, spaced 100 μ m apart, their line being perpendicular to the axis. When a particle passed cleanly through the beam illumination of some of the diodes was obscured and an electrical pulse was obtained whose magnitude was related in a known way to the size d of the particle. A flagging arrangement permitted the rejection of signals associated with particles which passed through the edge of the beam. A series of calibration experiments, employing stationary wires and also pellets of known size fired from a gun through the light beam illuminating the diode array showed that the values of d were accurate to ± 0.3 mm. The range of values of d that could be measured was 0.5 to 4.4 mm. The

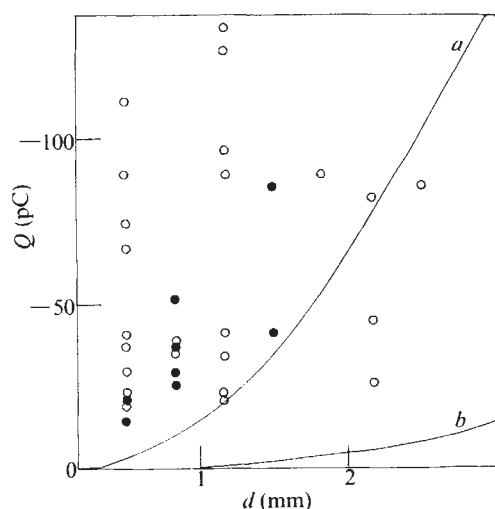
device is uncomplicated, functioned consistently well and (by integration of the d -pulses) provided crude but sensible estimates of precipitation rates. The tape-recorded commentary of the pilot showed that his observations of the presence or absence of raindrops coincided accurately with the information given by the device.

There was no difficulty in identifying Q/d coincidences from our tape-recorded charge and size pulses. The spacing in time of coincident Q/d pulses was typically one or two orders of magnitude shorter than the mean intervals between successive Q or d pulses. The measurements of electric field, particle size and particle charge were telemetered to ground, as was the pilot's commentary (which was very important in these studies) and other measurements. In 1976 much of the effort was devoted to optimisation of the various instruments carried on the Schweitzer, and most of the measurements were devoted to horizontal penetrations near the bases of thunderclouds, where the vertical field was generally negative (that is, in opposition to the fine weather field).

The great majority of precipitation particles were found to carry negative charges. Over extensive horizontal distances, with vertical fields up to about -30 kV m^{-1} , the volume charge density on precipitation ρp —obtained by summation of the individual charge pulses—was in the region of -5 nC m^{-3} . The corresponding precipitation current j was around 15 nA m^{-2} . These values of ρp and j are of sufficient magnitude⁵ and of the correct sign, to generate breakdown fields in the central regions of thunderclouds in the time generally considered to be available.

The number of coincident measurements of d and Q was limited because only about 20% of the particles entering the cylinder would pass through the light beam, and some insulation problems with the Q device were experienced in heavy rain. The coincidences obtained for two particular penetrations are displayed in Fig. 1. They show that no simple relationship exists between Q and d . Furthermore, the charges carried on the precipitation are usually far greater than the maximum (curve a) that they could acquire under an inductive mechanism operating even in regions where the field was at breakdown value. The charges on some of the smaller particles were close to the Rayleigh bursting limit and many of these hydrometeors carried charges in excess of those which would be required for levitation in positive fields of breakdown magnitudes.

Fig. 1 Measured coincident values of charge Q and size d of precipitation elements during two penetrations on 9 August 1976. $\circ$, Penetration 3; $\bullet$, penetration 4. Curve a from equation (1) with $E = 300 \text{ kV m}^{-1}$. Curve b from equation (1) with $E = 21 \text{ kV m}^{-1}$, which was characteristic of the field values measured during these penetrations.



It was not possible to make precise estimates of precipitation rate p from the d -pulses, because the sampling volume was small. It was established, however, that in regions where the values of ρp and j were large p did not generally exceed about 10 mm h^{-1} ; and on several occasions appreciable values of ρp were measured when p was below 1 mm h^{-1} . These observations of low precipitation rate were confirmed by the visual observations of the pilot.

Records from flights made on several days confirm our general findings—that precipitation currents and charge densities can be very high, even when precipitation rates are low, and that the individual values of Q are inexplicable in terms of the inductive theory. Thus it appears that precipitation may well play a major role in thunderstorm electrification, but that the mechanism responsible for the charge transfer remains to be identified.

A full discussion of these experiments will be described in a lengthy paper, currently in preparation.

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Pattern recognition applied to uranium prospecting

PATTERN recognition techniques provide one way of uniting quantitative and descriptive geologic data for mineral prospecting. A quantified decision process using computer-selected patterns of geologic data has the potential of selecting areas with undiscovered deposits of uranium or other minerals. When a natural resource is mined more rapidly than it is discovered, its continued production becomes increasingly difficult. For example, Lieberman¹ has noted that, although a considerable uranium reserve may remain in the U.S.A., the discovery rate for uranium is decreasing exponentially with cumulative exploration footage drilled. Pattern recognition methods of organising geologic information for prospecting may provide new predictive power as well as insight into the occurrence of uranium ore deposits. Often the task of prospecting consists of three stages of information processing: (1) collection of data on known ore deposits; (2) noting any regularities common to the known examples of an ore; (3) selection of new exploration targets based on the results of the second stage. Here we describe a logical pattern recognition algorithm that implements this geologic procedure to demonstrate the possibility of building a quantified uranium prospecting guide from diverse geologic data.

We chose to test prospecting by pattern recognition in an area with dimensions 85×90 km of the Colorado-Utah border between $108^{\circ}30'W$ to $109^{\circ}30'W$ and $38^{\circ}0'N$ to $38^{\circ}45'N$. This area contains many commercial uranium deposits as well as much unproductive area². Because this part of the Colorado Plateau is relatively well understood and explored, pattern recognition classification of barren and uranium-bearing areas may be verified by comparison with the results of past field exploration and development.

In the study area, uranium deposits occur chiefly as peneconcordant ore bodies in the Jurassic Morrison formation, a continental sandstone interbedded with mudstone. Most geologists agree with Hostetler and Garrels³ that the deposits were formed when uranium minerals precipitated from groundwater flowing through gently dipping sandstone beds with locally strong reducing capacity. Fossil plants and humic material seem the most likely sources of reducing agents. Uranium may have been derived from distant upland rocks, basement rock, overlying beds containing volcanic ash, up dip leaching of the host formation, or hydrothermal circulation associated with intrusives.

The principal recognition algorithm used here has been described in full elsewhere^{4,5}. In the present experiments, a computer searches geologic data for discriminants (called traits) that can be used to classify locations as either uranium-bearing or barren. In a training stage, the locations of known uranium deposits are presented to the computer together with geologic data at a number of sites in a study area. In the training district, the goal of data analysis is the identification of a set of geologic traits that discriminate producing areas from barren sites. This set of traits may then be taken to unexplored areas and used to predict the location of uranium-prone sites. Perfect classification, even of locations in a training district, is not expected because adequate geologic data may not be available, all ore bodies in a district may not have evolved similarly, some undiscovered ores may be incorrectly designated as barren, and instructions to the computer may not be optimal.

Many types of data, from standard geologic maps, geophysical, geochemical, and radiometric surveys and satellite imagery are now used in commercial exploration for uranium. In the present study, only readily available structural, stratigraphic, and geomorphic information was used in computerised prospecting. This information does not include many significant geologic features of uranium-bearing areas. Because major pre-ore structural features may have strongly influenced the flow of uranium-bearing groundwaters, however, this simple geomorphic information should have a considerable role in ore body location and will demonstrate the potential of pattern recognition techniques in prospecting.

Before analysing geologic data for patterns, several individual geologic features are tested to find some that are particularly characteristic of producing or of barren areas. The selection of features for recognition is based on estimates obtained from the training district of state-conditional probability density functions for the occurrence of features. Geologic data are simplified to binary-valued features, and a string of binary digits is used to describe each location (examples of the data available for this study are given in Table 1). The computer then uses this data base of binary features to contrast barren and producing areas by constructing more complex logical descriptions of these two classes of locations from the binary feature primitives. In this way, the computer may reveal logical relations particularly useful in uranium prospecting within large quantities of diverse geologic data.

Combinations of one, two and three binary values of features taken together constitutes traits. With N binary features

$$2N + 4C_N^2 + 8C_N^3$$

traits are examined, where C_N^M is the number of combinations of N things taken M at a time. With the 19 features of Table 1, for

Table 1 Examples of features for recognition of uranium deposits

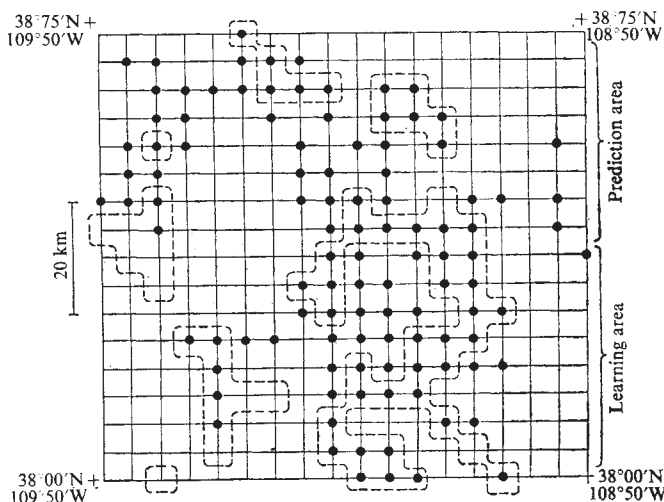
Position of location between nearest surrounding drainage divides
Lowest elevation within 3 km
Highest elevation within 3 km
Topographic gradient
Average elevation
Maximum bed dip within 5 km
Distance to Quaternary Sediment
Distance to major river
Distance to syncline axis
Distance to NE-trending basement fault
Distance to basement fault intersection
Distance to exposed crystalline rock
Number of formations outcrop within 2.5 km
Distance to anticline
Distance to Cretaceous intrusion
Is nearest anticline closer than nearest syncline?
Convergence or divergence of bed dip over Morrison formation
Does Morrison outcrop in 2.5 km?
Structure contour on Dakota sandstone base

example, no geologist could hope to examine all of the 8,474 possible traits. A computer is used to examine all these and to select the few traits most characteristic of barren and of producing areas. A trait characteristic of producing areas is selected if a trait occurs at more than K_1 producing locations and fewer than K_1^* barren locations; similarly, K_2 and K_2^* define characteristic traits of barren areas. In the present experiments, K_1 , K_1^* , K_2 , and K_2^* were about 60%, 10%, 40%, and 10% of producing and barren areas, respectively. In a final classification stage, if the number of producing traits exceeds the number of barren traits present at a point, the point is classified as uranium-bearing by the computer, otherwise it is classified as barren.

We tested how well the computer can learn in one region and predict in another in a simulated exploration survey. The computer was given geologic data and the locations of known mines in the southern part of our grid. After surveying possible features and developing traits on 162 points in the southern part of our grid, geologic data for 144 points in the northern part of the grid were given to the computer with instructions to select the most likely uranium prospects based on results of learning in the south. Results of this recognition and prediction are given in Fig. 1.

In the training area, 80% of producing points and 82% of barren points were correctly recognised. Most misclassified barren points are adjacent to known deposits, so that these

Fig. 1 Results from Experiment 1. The computer was trained on the southern part of the grid and predicted in the north. Grid points classified uranium-bearing on the basis of traits are indicated by ●. Grid points with known uranium production are within the dashed contour (production data from Williams, 1964).



errors are not unreasonable. With a 5-km grid spacing, some errors are expected as adjacent grid points may have similar descriptions in our limited feature space.

In the north, despite the small training sample and the limited information available in features, the computer did pick as good prospects some points in each of five producing zones. Correct recognition rates in the north are comparable with those in the south, 83% for producing areas and 74% for barren areas. Since no set of locations will resemble the training set as much as the training set itself, an error rate in prediction comparable with that in learning may be regarded as excellent performance. Also, some of the errors in classifying barren points may represent undiscovered deposits.

To further test the method, training and prediction was carried out for the entire area of Fig. 1. This differs from the procedure of learning in one area and predicting in another, and suggests using pattern recognition to identify overlooked deposits within a mining district. Results comparable with those from our Experiment 1 were obtained, attesting to the stability of the method against large variations in the amount of learning material. We gain additional confidence in computerised trait selection because traits are always geologically reasonable, though it is not obvious before applying our procedure which features will be combined into traits for optimum recognition.

To demonstrate that the geologic patterns found by our procedures are not random accidents, control experiments to test for self-deception were performed. Although not a part of standard pattern recognition procedures, some such test is important when considering complex geologic systems. The computer was given spurious but realistic geologic information by holding geologic data at each grid point fixed but rotating the spatial classification pattern to be duplicated by 180°. This rotated pattern retains the spatial distribution and degree of order of the original classification. In this experiment, all 306 grid points were again used as a training set. No traits were found to satisfy the K/K^* conditions imposed on traits from real data. Relaxing the K/K^* conditions to obtain traits, the algorithm could force this artificial data barely above the 50% correct recognition rate expected from random binary selection of prospects. The failure of recognition based on spurious but spatially realistic information (as well as similar failures of recognition based on randomly generated binary features) lends confidence to results based on real data.

In these and related experiments, a contextual/decision theoretic pattern recognition procedure was able to extract significant logical relationships between diverse types of geologic data and to use these relationships in correct recognition of uranium-producing areas. Correct recognition rates up to 85% for uranium-producing areas with few false positives indicates the potential of pattern recognition in selecting exploration targets. When comprehensive data from standard geologic maps, LANDSAT imagery, geochemical, radiometric, and geophysical surveys are available, computerised prospecting procedures may provide a powerful new approach to predicting new areas of commercial resource potential. A complete description of this work will be published elsewhere.

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An important ground surface sink for atmospheric nitrous oxide

ONE of the more remarkable achievements of atmospheric chemistry is the identification by Crutzen¹, in 1970, of nitric oxide (NO) as the main natural agent for the destruction of ozone produced photochemically in the stratosphere. The principal source of stratospheric NO is the reaction of excited oxygen atoms ($O(^1D)$) with nitrous oxide (N_2O)², present as a normal constituent of the Earth's atmosphere. N_2O was first observed, in 1938, by Adel³ in the absorption bands in the 7.8 μm region of the solar spectrum, but knowledge of its atmospheric life cycle is still very incomplete because of the few measurements available of its atmospheric concentration and limited information on atmospheric sources and sinks. The soil has been suggested as a major source of atmospheric N_2O ⁴ and the ocean has been proposed to act both as a source⁵ and as a sink⁶. The only sink so far positively established by atmospheric measurement lies in the stratosphere, being predominantly photodissociative in nature with an additional contribution from the chemical reaction with excited oxygen atoms^{4,7}. We report here evidence for the existence of an appreciable, but unidentified, ground surface sink mechanism.

Interest in monitoring atmospheric N_2O has been stimulated recently, both from realisation of its important role in ozone chemistry, and concern that perturbation of the ozone level in the stratosphere may occur due to enhanced N_2O emissions from the soil arising from increasing world-wide application of nitrogenous fertilisers. McElroy⁸ has predicted that a 20% reduction in the concentration of stratospheric ozone may occur by the first quarter of the twenty-first century if present trends in the use of nitrogenous fertiliser continue.

The recent development⁸ of the heated electron capture detector for gas chromatographic determination of N_2O has facilitated the measurement of ambient levels of N_2O in air without the need for previous concentration of the air sample^{9,10}.

In the following, we describe some measurements of N_2O made using such a detector at AERE Harwell in July and August 1976. Outside air was sampled at 5 m above ground level and drawn through a 6 feet stainless steel tube into a 5 cm³ sample loop. The air in this loop was injected every 2 h into a PYE 104 gas chromatograph equipped with an electron capture detector operating at 350 °C. A 9 feet Porapak QS column, maintained at 27 °C, was used to separate N_2O using a 90% Ar–10% CH₄ mixture as carrier gas flowing at 30 cm³ min⁻¹. The apparatus was operated continuously for a five-week period commencing 7 July and ending on 9 August; our data was obtained in a period of extremely stable operation from 24 July to 8 August. This was demonstrated both by negligible changes in observed standing current of the detector ($1.00 \pm 0.03 \times 10^{-9}$ A) and by minimal changes in the signal displayed by injecting a standard sample at regular intervals. (0.278 ± 0.005 p.p.m.). During the same period outside air samples gave values ranging from 0.233 to 0.315 p.p.m.

The most important aspect of the data, shown in Fig. 1, is the observation of a regular diurnal variation of N_2O concentration on each of the 11 days shown. A minimum was nearly always recorded in the early hours of the morning and a maximum was recorded in the late afternoon or evening. Since the amplitude of this diurnal variation was only about 10% of the mean value, we have taken particular care to establish that it was not due to diurnal changes in instrument sensitivity associated either with changes in instrument conditions such as ambient temperature, or with changes in the concentrations of other atmospheric species such as water vapour or carbon dioxide. The observations, therefore, seem to indicate a real physical phenomenon which can only be explained either by some process which increases the N_2O concentration during the day, or alternatively depletes the N_2O concentration at night. Pierotti and Rasmussen¹¹ have noted that the N_2O concentration

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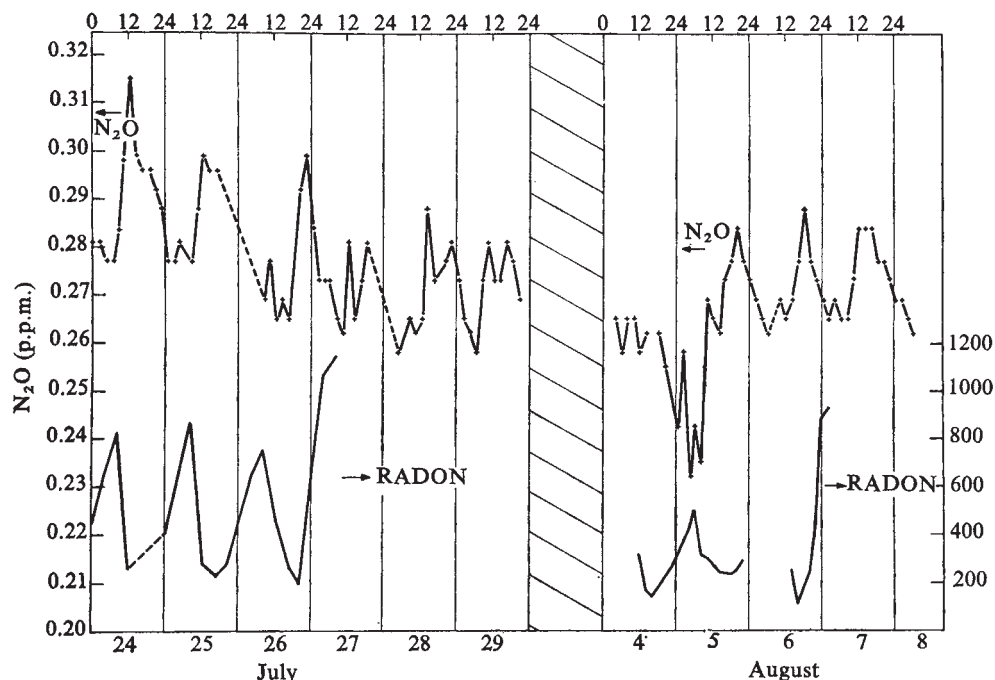


Fig. 1 Daily variation of N_2O and radon from 24 July to 29 July 1976, and from 4 August to 8 August 1976 (---, data pattern uncertain).

in power plant plumes is increased above the background level, but there is no reason why such combustion processes should lead to regular increases of the type shown in Fig. 1.

It seems rather that N_2O exhibited diurnal behaviour typical of gases such as sulphur dioxide, ozone and peroxyacetyl nitrate (PAN) which are absorbed at ground level surfaces^{12,13}. Measurements of such gases made near the ground show a reduction in concentration at night, as they become depleted in the shallow layer of stable air which develops below the nocturnal inversion, and a rise during the day, as solar radiation gradually breaks up the inversion and allows fresh material to mix downwards. Inversions developed on many nights during the observational period which was characterised by anticyclonic weather conditions and clear skies.

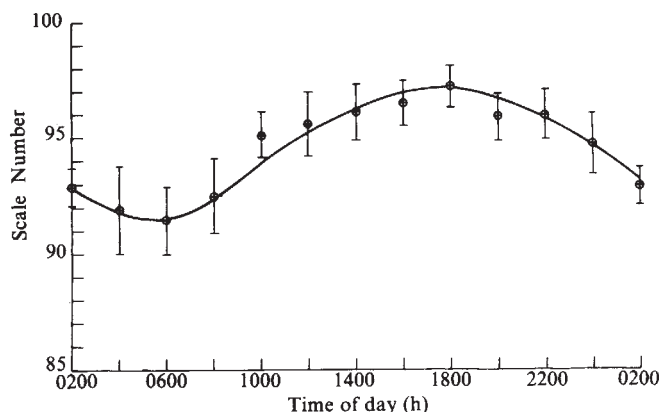
Such diurnal behaviour is anti-correlated with that of radon¹⁴ which is emitted rather than absorbed, at the ground surface. Included in Fig. 1 are the atmospheric radon concentrations, also measured at Harwell with continuous monitoring equipment, which showed this strong anti-correlation with the N_2O observations on several days during the period. A possible alternative explanation would be a gas-phase reaction with another gas released at ground level, but this is unlikely with a gas as unreactive as N_2O and no suitable candidate reactions have been suggested in the literature. Thus we conclude that absorption of nitrous oxide, probably by vegetation or the soil, is the most likely explanation of our observations though the mechanism by which this occurs is not clear.

Matsubara and Mori¹⁵ suggested that N_2O would be removed by soil microorganisms and some recent investigations by Blackmer and Bremner¹⁶ provides experimental evidence that soils can possess more potential to act as a sink than as a source of N_2O in conditions favourable to denitrification. These observations indicate that a sink other than destruction in the stratosphere may be significant for atmospheric N_2O . If the deposition is occurring irreversibly then the size of this sink can be estimated from the deposition velocity (v_g), which is related to the exponential decay constant λ , governing the rate of concentration decrease by the simple expression $\lambda = v_g/h$ where h is the height of the stable layer from which deposition is occurring^{13,17}.

Figure 2 illustrates the average diurnal pattern for the period in question. This was derived by assigning a value of 100 to the maximum N_2O concentration observed during any 0000–2400 cycle and scaling the other measurements accordingly. For the

days considered, the scale numbers were then averaged for each respective sampling time. The average value of λ for decay of N_2O between 1800 h and 0600 h can be derived from Fig. 2, using a value of 0.280 p.p.m. for the mean N_2O concentration during the period. The magnitude of h is unknown, but, since it is the same for all gases undergoing deposition, the v_g for N_2O can be obtained directly by comparison with data on the average diurnal variation of ozone at Harwell over the same period. The value of λ for N_2O from Fig. 2 is $(5 \pm 0.7) \times 10^{-3} \text{ h}^{-1}$ and the corresponding λ for ozone is $(0.12 \pm 0.02) \text{ h}^{-1}$. Since the average deposition velocity for ozone observed in similar conditions is about 0.3 cm s^{-1} ¹⁸ we calculate a value for N_2O of $1.25 \times 10^{-2} \text{ cm s}^{-1}$. If, in the absence of further information, we assume this value is typical of all land surfaces then we can estimate the magnitude of the global sink for N_2O . The mean atmospheric lifetime of a species being removed by deposition at the Earth's surface is equal to H/v_g , where H is the equivalent height of the atmosphere (8.3 km) and v_g is the mean deposition velocity for the earth's surface. Assuming that deposition occurs all year at the same rate but only on land surfaces (0.26 of the total surface) then the life time of N_2O due to this sink is 8.1 yr. Taking a mean mixing ratio for N_2O of 0.3 p.p.m.¹⁹,

Fig. 2 Average diurnal variation for N_2O . $\oplus$, Average scaled number value for corresponding injection time; I , ± 1 standard error of the mean.



the magnitude of this sink is estimated at 320 Mt yr^{-1} . This figure, which can only be very approximate at this stage, is twice the current estimated combined ocean source, 135 Mt yr^{-1} ⁵, and soil source, 25 Mt yr^{-1} ⁴. It is more than an order of magnitude greater than the figure of 13 Mt yr^{-1} for the stratospheric sink²⁰. Use of the mean lifetime figure of 8.1 yr would substantially reduce McElroy's estimate of the reduction in stratospheric ozone due to the use of nitrogenous fertilisers.

Further data in a variety of conditions are needed before the global magnitude of this sink mechanism, which seems larger than any other, can be estimated with confidence.

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Generation of a supported iridium catalyst of extremely high dispersion

THE preparation of supported metallic catalysts in a very high state of dispersion—that is, with a very small average metallic particle size—is of considerable current interest. Not only is the surface area per unit mass of metal thus increased, but if the metallic particles are sufficiently small, their intrinsic catalytic properties may be different from those of the massive metal. On the whole, changes of the latter sort seem to be found when the particle diameter is less than about 2 nm (refs 1, 2). The use of a metallic cluster carbonyl (MCC) as a means of preparing a dispersed metallic catalyst has been the subject of preliminary exploration^{3,4,5}. Although it was indicated that this route could yield highly dispersed metal, it was clear³ that considerable cluster aggregation occurred during thermal decarbonylation and hydrogen reduction (at 573 K). This note reports the preparation from $\text{Ir}_4(\text{CO})_{12}$ of a supported iridium catalyst in a very high state of dispersion.

The preparative details are: the support (Wohlm γ -alumina, $200 \text{ m}^2 \text{ g}^{-1}$) vacuum-dried at 380 K for 15 h, was impregnated using the method of incipient wetness with a solution of $\text{Ir}_4(\text{CO})_{12}$ used as obtained from Alpha Chemical Inc in cyclohexane (0.4 mg cm^{-3} , AR grade saturated). After standing for a few hours, the solvent was removed under vacuum at room temperature, and the sample was then heated to 623 K under vacuum for 4 h. The sample was then heated in hydrogen at 623 K for 15 h, and was then evacuated to better than 1 mPa at the same temperature. At this point, the catalyst was ready for characterisation by gas adsorption (H_2 or CO) or by

electron microscopy. Catalyst compositions were determined by X-ray fluorescence.

The results for catalyst samples with loadings of 0.31 and 2.48 wt % iridium (dry weight basis) are given in Table 1. The extent of gas uptake over the pressure range 0.3–3 kPa was virtually independent of pressure and is taken to correspond to monolayer adsorption. In the case of iridium it is reasonable to take the monolayer chemisorption stoichiometry for hydrogen, $X_m(\text{H}_2) = 2$ ⁽¹⁾, that is each surface iridium atom carries one hydrogen atom at monolayer coverage. On this basis the average iridium particle diameters were computed which are also included in Table 1 for comparison with the electron microscopic data.

Table 1 Characterisation of supported iridium catalysts

	Gas adsorption, ‡ 293 K 0.3–3 kPa (10^{21} molecules per g Ir)		$N_{(s)H}/N_{(T)Ir}^\dagger$	$N_{(s)CO}/N_{(T)Ir}^\dagger$
	H_2	CO		
$\text{Ir}/\gamma\text{-Al}_2\text{O}_3$ 2.48 wt % Ir	0.67	1.45	0.43 (2.5 nm)*	0.46
$\text{Ir}/\gamma\text{-Al}_2\text{O}_3$ 0.31 wt % Ir	1.51	7.66	1.0 (<1.1 nm)*	2.44

* Average iridium particle diameter obtained from $N_{(s)H}/N_{(T)Ir}$ and $X_m(\text{H}_2) = 2$.

† Obtained using the result that 1 g Ir contains 3.13×10^{21} atoms: $N_{(s)}$ and $N_{(T)}$ number of atoms at surface and in total respectively.

‡ Corrected for adsorption on the support.

Two main conclusions emerge from Table 1. For the catalyst for which $N_{(s)H}/N_{(T)Ir}$ (which equals the metallic dispersion $D = N_{(s)Ir}/N_{(T)Ir}$ by virtue of the value assigned to $X_m(\text{H}_2)$) is less than unity, there is reasonable agreement between $N_{(s)H}/N_{(T)Ir}$ and $N_{(s)CO}/N_{(T)Ir}$ at monolayer coverage, indicating $X_m(\text{CO}) \approx 1$. However, in the case of the catalyst with the lower iridium loading of 0.31 wt % Ir for which $N_{(s)H}/N_{(T)Ir} = 1$, the value of $N_{(s)CO}/N_{(T)Ir}$ indicates $X_m(\text{CO}) = 2.44$. Although it is known that for extremely highly dispersed platinum the monolayer chemisorption stoichiometry ($X_m(\text{H}_2)$) may fall somewhat below two², we have not observed similar behaviour with extremely highly dispersed iridium. Bearing in mind that $D = 1.0$ is consistent with a range of particle sizes below a limit of about 1.1 nm in the case of iridium, and also noting that for this catalyst we failed to resolve any metallic particles electron microscopically, it is clear that the average iridium particle size was extremely small, certainly <1 nm. Since one would expect a range of particle sizes, it is reasonable to expect that some proportion of the iridium was retained as Ir_4 clusters. It remains to be assessed whether $N_{(s)CO}/N_{(T)Ir} = 2.44$ indicates that this proportion was substantial. Comparison with some results of Della Betta⁶ for highly dispersed ruthenium catalysts suggest that the answer is probably in the negative. Della Betta studied the adsorption of H_2 and CO for average Ru particle sizes in the range 1.1–2.5 nm (evaluated from H_2 chemisorption data, and assuming $X_m(\text{H}_2) = 2$); the corresponding values for $N_{(s)CO}/N_{(T)Ru}$ were in the range 2.3–3.8. Clearly, with a metal for which a molecular cluster carbonyl exists ($\text{Ru}_3(\text{CO})_{12}$ in the case of Ru), $N_{(s)CO}/N_{(T)M}$ can approach a value characteristic of the MCC even though the metallic particle size may be rather larger than that existing in the MCC itself.

Comparison with the results of Smith *et al.*⁴ suggests that unaggregated metallic cluster skeletal units generated from metallic cluster carbonyls, cannot be retained on these supports if the temperature rises substantially above room temperature. This is probably a consequence of the facile migration of these units across these support surfaces at elevated temperatures. It may prove possible to overcome this problem by designing supports with surface sites of high binding energy at which the metallic cluster skeletal units may be immobilised, or to

restrict skeletal unit migration by controlled pore structure of the support. These approaches are currently under investigation.

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Hydrogen in the Earth's core

THE density of the Earth's core is 8–10% less than that of pure iron at the same pressure and temperature¹ and this is usually interpreted as implying the existence of a substantial amount of light element(s) in the core. Many possibilities have been considered¹, with sulphur and oxygen² receiving most recent attention, but in this note the discussion is limited to hydrogen's contribution to the density deficit. It is shown that hydrogen is not only effective in decreasing the density but is also highly soluble in iron in the relevant conditions. Only about 1% by mass of hydrogen is required in the Earth's core to explain the density deficit, and this corresponds to a hydrogen-iron mass ratio that is only about one-tenth of that present in type I carbonaceous chondrites³. The significance of hydrogen as a contributor to the density deficit is, therefore, determined by the extent to which the Earth-forming matter consisted of low temperature condensates.

Hydrogen was prominent in the earliest discussions of core composition^{4,5} but since it enters iron as interstitial atoms, Ringwood⁶ proposed that it could not significantly contribute to the density deficit. In fact, the interstitial hydrogen substantially expands the host metal, and the zero pressure hydrides of transition metals are typically 10–20% less dense than the pure metal⁷, in contrast to ionic or covalent hydrides. This has frequently been interpreted as the formation of the large (but highly compressible) H⁻ ion⁷, but the thermodynamics are better understood in terms of a screened proton model⁸, according to which the hydride can be thought of as an alloy of the transition metal and metallic hydrogen.

For simplicity, consider a host metal characterised by rigid ion cores of size r_c and effective charge Z (not necessarily an integer) immersed in an essentially uniform compensating electron gas. The energy per ion of the alloy $M_{1-x}H_x$, where M is the host metal, is then

$$E = (1-x)E_M(V_M) + xE_H(V_H) + \delta E_{MH} \quad (1)$$

where V_M is the volume occupied by Z conduction electrons, $V_H = V_M/Z$, E_M and E_H are the energies per ion of the pure host metal and pure metallic hydrogen⁹ respectively, and δE_{MH} is a small correction term (which includes the M -H interaction). Neglecting δE_{MH} , the small x limit gives

$$x \approx \frac{1}{\rho} \left(\frac{\partial \rho}{\partial x} \right)_P \approx \frac{P - P_H - B}{ZB} \quad (2)$$

where ρ is the mass density, P is the pressure, B is the host metal bulk modulus, and P_H is the pressure that metallic hydrogen would have if it had the same conduction electron density as the host metal. At pressures less than a few megabars, $P_H < 0$

and $B \gg P$ or $|P_H|$, so $\alpha \approx -1/Z$, and the model approximately predicts the observed density decrease.

According to the empty core pseudopotential model¹⁰, the known equation of state for iron¹¹ is consistent with $Z \approx 2$ near $P = 0$ and $Z \approx 3$ at Mbar pressures, both with $r_c \approx 0.49 \text{ \AA}$. More realistic models for iron yield similar results for α . Since $|\alpha|$ decreases as P increases, the hydride is more compressible than the host metal, but not to such an extent as to be incompatible with the known pressure-density relationship in the Earth's core. An alloy with composition similar to FeH would explain the observed core density. Inclusion of δE_{MH} in the calculation actually decreases the required amount of hydrogen slightly. Thomas-Fermi calculations¹² predict that a much larger amount of hydrogen is needed, essentially because that method overestimates the conduction electron density.

Just above the iron melting point, the hydrogen solubility⁸ for $x \ll 1$ is $x \approx 0.015 P^{1/2}$, where P is in bars, so a pressure of a few kbar suffices to form an FeH composition. At much higher pressures, where the coexisting molecular hydrogen ceases to be an ideal gas, the hydride should form even at $T = 0 \text{ K}$, because the enthalpy of molecular hydrogen increases more rapidly than that of metallic hydrogen⁹. In the low temperature limit

$$x \approx \exp(-\Delta H/k_B T) \quad (3)$$

where ΔH is the enthalpy of hydride formation per proton and T is the temperature. At $P = 0$, $\Delta H = \Delta H_0 \approx 0.25 \text{ eV}$ (ref. 8). At $P \neq 0$, equation (1) with $\delta E_{MH} = 0$ implies

$$\Delta H \approx \Delta H_0 + P \left(V_{e1} - V_{H,mol} - \frac{P_H}{B} V_{e1} \right) \quad (4)$$

where V_{e1} and $V_{H,mol}$ are the volume per conduction electron in the hydride and volume per proton in the molecular hydrogen phase, respectively. From the known properties of molecular and metallic hydrogen⁹, $\Delta H < 0$ if $P > 200 \text{ kbar}$. Allowances for $\delta E_{MH} \neq 0$ and finite temperature in the Earth's core do not significantly alter this prediction for hydride formation. At pressures much higher than that in the Earth's core, hydrogen may again become insoluble¹³.

The proposed hydride would be an excellent conductor, perhaps even better than pure iron (since metallic hydrogen conducts electricity or heat over 10 times better than iron at the same pressure and temperature¹⁴). It would also have a lower melting point than pure iron, and other properties which are compatible with present knowledge of the Earth's core.

If at least 10% of the Earth-forming material was a low temperature condensate (containing hydrogen in the form of water) as many models require^{1,2}, then the proposed core composition is possible. The extent to which hydrogen (or water) was lost during the heating accompanying Earth formation depends on the detailed pressure and temperature history. If, for example, the water reacted with iron at a pressure such that most of the released hydrogen could enter in solution with iron, then volatile retention could be high. The decreasing abundance of water with increasing metamorphic grade in chondrites is not an argument against volatile retention, since the hydrogen is only retained at high pressure, and would be expelled if the pressure were subsequently released. In conclusion, hydrogen is conceivably the major cause of the density deficit in the Earth's core, but is most probably just one of several contributing causes.

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Geochronology of some blueschists from Ile de Groix, France

THE Ile de Groix series consists of metavolcanic and metasedimentary rocks, with or without glaucophane. These rocks belong to a South Brittany polymetamorphic complex which is named the 'domaine de l'Anticlinale de Cornouaille' by Cogné¹. In this complex, anatectic granites occur in the midst of a migmatitic and gneissic terrain^{1,2}. Some metasedimentary and metavolcanic rocks which occur in the terrain are cut by Hercynian granites. The Ile de Groix series^{1,3,4} is characterised by high pressure mineral assemblages with glaucophane, jadeitic pyroxene and lawsonite⁵—these assemblages are unknown on the adjacent mainland. The series is considered to have formed during two metamorphic periods: the first produced high pressure–low temperature mineral assemblages and the second produced a recrystallisation of mineral assemblages in greenschist facies⁶. In this note, we shall present new isotopic age data on various rocks and minerals from Ile de Groix, hoping to delineate the time sequence of original magmatism, sedimentation and subsequent metamorphism.

Six phengitic mica samples were separated from metavolcanic rocks and three from possibly metatuffaceous sediments. They were analysed for Rb and Sr concentration and Sr isotopic compositions. The ages calculated from five whole rock–phengite pairs, two epidote–phengite pairs and two 0.705-phengite pairs are clustered in the range of 340 to 375 Myr ($\lambda^{87}\text{Rb} = 1.42 \times 10^{-11} \text{ yr}^{-1}$) (Fig. 1). K/Ar measurements, by J. C. Hunziker, on four of these samples yield slightly lower ages (340 to 360 Myr), but are, nevertheless, within error of the Rb–Sr ages (Fig. 1); a paragonite gives 273 ± 14 Myr.

The mica ages could be interpreted as representing either the time of crystallisation or cooling⁷, but the actual scatter of these age data suggests the effect of cooling. Thus the age of 375 ± 15 Myr is considered as a minimum for the crystallisation of phengitic mica and thus also for the time of high pressure metamorphism. The paragonite age is interpreted as the final cooling event.

This interpretation does not agree with Carpenter and Civetta⁸. They obtained K–Ar ages on three glaucophanes between 315 ± 11 and 329 ± 33 Myr and on four phengites between 327 ± 10 and 339 ± 10 Myr. The concordance of these results led them to conclude that both minerals were formed at the same time about 335 ± 20 Myr ago during an episode of high pressure metamorphism.

Their data, however, as well as our results, show that phengites give systematically older ages than glaucophane. An independent measurement by H. Maluski⁹ by the $^{40}\text{Ar}/^{39}\text{Ar}$ method has also confirmed the lower age of glaucophane (320 ± 8 Myr) compared with phengites. This difference is significant and consequently the ages of 320 ± 8 and 335 ± 20 Myr cannot represent the time of high pressure metamorphism.

This relation between glaucophane and cogenetic phengite has also been found by Coleman and Lanphere¹⁰, Suppe and Armstrong¹¹ in the Californian blueschists and by Hunziker¹² in Alpine high pressure metamorphic terrains. These authors suggest that glaucophane has lost argon during later metamorphic or tectonic events, and that glaucophane ages may postdate high pressure metamorphism.

Such an interpretation seems to apply to the data from Groix; glaucophanes are often aligned parallel to early isoclinal fold axes, probably synchronous with high pressure metamorphism, but subsequently, they often became re-oriented about axes of later folding and underwent recrystallisation. This suggests that 320 Myr glaucophane ages, in fact, record the effect of this later folding phase, and that the high pressure metamorphism occurred at, or before 375 Myr ago.

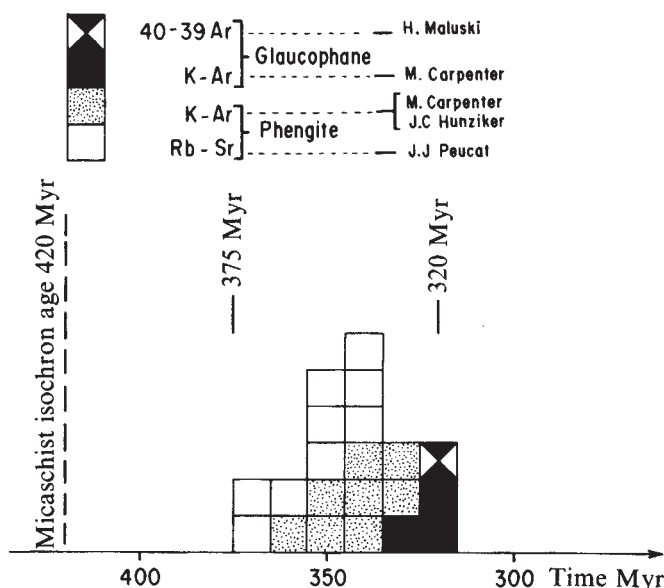


Fig. 1 Rb/Sr and K/Ar ages for phengites and glaucophanes. $^{40}\text{Ar}/^{39}\text{Ar}$ step release age for glaucophane.

Whole rock Rb–Sr isotopic data on four micaschists have been previously published by Vidal¹³. New measurements on two micaschists without glaucophane and on two with glaucophane as well as these four data of Vidal allow us to construct a reference isochron with an age of 420 Myr (Fig. 2).

Among the basic igneous rocks, one data point (Fig. 2) (an alkali-rich rock characterised by the presence of abundant phengitic mica: 2444) also lies near, or on, the micaschist isochron. However, most metabasaltic rocks have very low Rb/Sr ratio (<0.25) and as their $^{87}\text{Sr}/^{86}\text{Sr}$ ratios lie between 0.7052 and 0.7101, they do not allow construction of an isochron. Assuming an age of 420 Myr for the time of metamorphism, the calculated initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios range from 0.705 to 0.709. Reasons that might explain this scattering are: possibly seawater alteration effects, contamination from sediments, heterogeneous igneous source material with large differences in initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. Preliminary chemical data on major and trace elements suggest that the original source rocks are produced from alkali-basalts and tholeiites, but higher initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios values suggest possible contamination.

We do not think that this age of 420 Myr reflects the sedimentation age because this would certainly have been disturbed by two metamorphic episodes^{14,15}. Near Groix, very similar protoliths (Pouldu series) are cut by the Moëlan granite¹⁶ dated at 474 ± 2 Myr ($\lambda^{87}\text{Rb} = 1.42 \times 10^{-11} \text{ yr}^{-1}$) by Vidal¹³. Sedimentation here, therefore, pre-dates this Ordovician age.

Assuming that the 320 Myr age on glaucophane reflects the effects of a later metamorphic and deformation event,

it is possible to associate this event with retrograde metamorphism. Consequently, the age of 420 Myr could reflect the high pressure–low temperature metamorphism, or a previous episode of metamorphism, or dewatering of sediments.

A pre-high pressure–low temperature metamorphism is possible, but is not recognised from petrographic study. When we take into account that the age 420 Myr is deduced from a reference isochron and that the 360–375 Myr mica ages are cooling ages, then the difference between these two ages does not necessarily imply two separate periods of metamorphism. Hence we interpret the age of 420 Myr as representing the time of high pressure–low temperature metamorphism, or possibly, the time of dewatering of sediments.

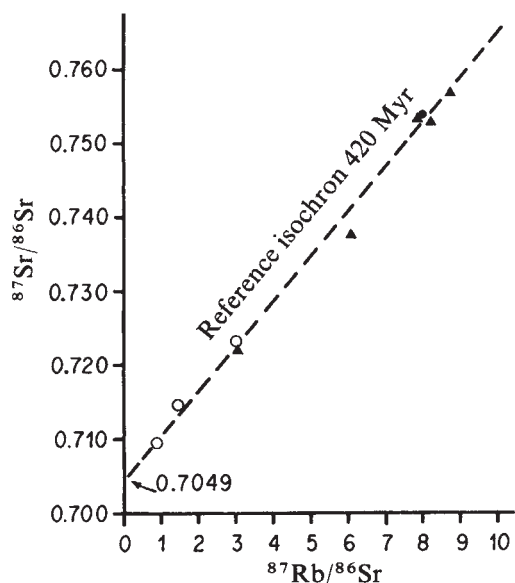


Fig. 2 Whole-rock reference isochron for micaschists: (○) micaschists with glaucophane, (△) micaschists without glaucophane. Age calculated from seven points is 421 ± 7 Myr with an $^{87}\text{Sr}/^{86}\text{Sr}$ initial ratio of $0.7049 \pm 3.10 \times 10^{-4}$ (λ $^{87}\text{Rb} = 1.42 \times 10^{-11} \text{ yr}^{-1}$). (●) Micaceous metabasic rock (2444).

In conclusion, the following metamorphic episodes can be delineated: (1) Pre-Hercynian period is characterised by the advent of high pressure–low temperature metamorphism about 370–420 Myr ago. The limits of this metamorphic period in South Brittany can be constrained by the study of Morbihan migmatites. The migmatisation affects an orthogneiss dated at 462 ± 7 Myr by Peucat and culminates with emplacement of anatectic granites at 376 ± 12 Myr (ref. 17). High pressure metamorphism and migmatisation events correspond with an important pre-Hercynian orogeny, possibly with paired metamorphic belts of *circum* Pacific type, in which the basement was formed. (2) The Hercynian period produced partial retrogression of high pressure mineral assemblages into greenschist facies causing loss of argon in glaucophane. The continental basement was also affected by this retrograde metamorphism, especially near folds and shear zones. It is characterised by major plutonism *ca* 340–290 Myr ago. The thermal history of this area ends with cooling beneath 300°C 290 Myr ago (Rb/Sr mica ages throughout south Brittany give 280–290 Myr) (ref. 18).

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⁴⁰Ar/³⁹Ar ages of a diabase sill and a basalt in the Central-Pacific Basin

THE deep sea drilling project (DSDP) Leg 17 has covered the area of the Central-Pacific Basin south of the Mid-Pacific Mountains and east of the Marshall Islands. Since this region belongs to the magnetic quiet zone, no definite ages of oceanic crust could be assigned. Results of drilling at Site 166 ($3^\circ 45.7'\text{N}$, $175^\circ 4.8'\text{W}$) and at site 167 on Magellan Rise ($7^\circ 4.1'\text{N}$, $176^\circ 49.5'\text{W}$) have given estimates of crustal ages of about 120 and 135 Myr respectively from the basement fossils, which suggests that ages increase northward on this set¹. Furthermore, the parallelism of linear bathymetric features around site 167 to the magnetic and bathymetric trends around site 166 has been interpreted by Winterer¹ to mean that they belong to the same spreading ridge system with an average spreading rate of 2–4 cm yr⁻¹ for the crust between the two sites. Since sites 169 and 170 are located to the north-west of the sites 166 and 167 in the magnetic quiet zone, they were expected to give older ages, probably Jurassic, from the extrapolation of the trend stated above and by referring to the time scale of magnetic reversals². It has been revealed, however, that at site 169 ($10^\circ 40.2'\text{N}$, $173^\circ 3.0'\text{E}$) the oldest sediment recovered yields Albian age (100–104 Myr), which overlies basalt. At this site, about 8 m of diabase sill was drilled, which intruded Turonian (87–95 Myr) or Cenomanian (95–100 Myr) sediments³. At site 170 ($11^\circ 48.0'\text{N}$, $177^\circ 37.0'\text{E}$), the oldest sediment overlies the basalt is also of Albian age⁴. These ages are much younger than those expected from ages obtained at sites 166 and 167, which may reflect the different history of the ocean floor from those at sites 166 and 167. To clarify this point, two igneous rocks were dated by ⁴⁰Ar/³⁹Ar method. Details of experimental procedures are described elsewhere⁵.

The sample 169-6-2 is a diabase sill drilled at site 169 and relatively fresh among drilled samples, while the sample 170-16-2 is a basalt drilled at site 170, which was the lowermost one at this site. Results of ⁴⁰Ar/³⁹Ar dating are shown in Fig. 1a and b.

As shown in Fig. 1a, for the sample 169-6-2 the plot of the highest temperature fraction is clearly deviated to the higher ⁴⁰Ar/³⁹Ar ratio from the isochron, which indicates the existence of excess ⁴⁰Ar in this fraction. Excluding this point, however, other points form a good isochron of 90 Myr with the MSUM value of 1.428. This age is nearly

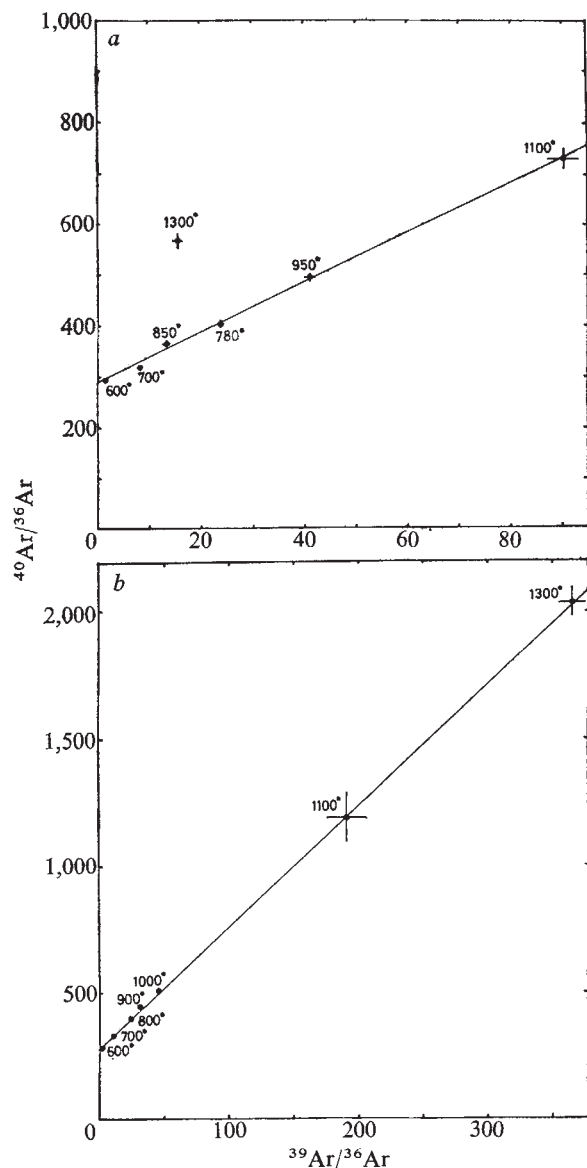


Fig. 1 $^{40}\text{Ar}/^{39}\text{Ar}$ isochron diagrams for Leg 17 samples. Samples were heated for one hour at each temperature which is indicated by figures at each plot. Horizontal and vertical bars at each plot indicate error range (one standard deviation) for each analysis. Bern 4M muscovite (18.5 Myr) was used as an age standard. (All necessary corrections including those for interference products have been made.) (a) Sample Leg 17-169-6-2; age: 90.0 ± 3.5 Myr; intercept: 285.4 ± 3.5 , (b) Sample Leg 17-170-16-2; age: 97.2 ± 2.5 Myr; intercept: 280.3 ± 2.1 .

the same or a little younger than that of intruded sediments. Petrologically the diabase has been regarded to have intruded as a sill rather than extruded as a flow³. Our results agree with this observation.

The ^{40}Ar - ^{39}Ar isochron age of the basalt 170-16-2 is 97 Myr (Fig. 1b) and a little younger than that of the overlying sediments which contain late Albian radiolaria⁴. These basalts are reported to be altered⁴, but stepwise-heating result has shown that it causes apparently no serious effect on the obtained age. The MSUM value for this sample is 1.605. Results for the sample 170-16-2 supports the middle Cretaceous age of basalt as estimated from the overlying sediments, which is much younger than Jurassic age which was expected. There are two possible explanations for this difference.

First, the basalt drilled at site 170 may be only a sill and not represent the true basement whose age is older than that obtained in this study. Bass *et al.* has interpreted the

basalt as a sill of ocean island tholeiite from textural evidence and lack of glass or pillow structure⁵. In order to explain the existence of the magnetic quiet zone in this region, the crustal age should be in Jurassic and older than about 155 Myr, considering a long period of normal geomagnetic polarity at that time².

Secondly, the sample 170-16-2 may represent the basement rock, and the oceanic crust in this region might be really young—of middle Cretaceous age. Although the $^{40}\text{Ar}/^{39}\text{Ar}$ isochron age obtained for present sample is a little younger than that estimated for the overlying sediments, the difference may not be significant, considering its error range. Since a long period of normal geomagnetic polarity in Jurassic began about 108 Myr ago², the crustal age should be nearly the same or younger than about 108 Myr in order to explain the existence of the magnetic quiet zone. In addition to the age data, estimated from the lowermost sediments at sites 169 and 170, our present result is compatible with this interpretation. In this case, the crust in this region might be characterised by recurrent flows. In fact, many sills and/or dykes have been observed in the Mid-Atlantic Ridge area⁷ and recurrent flows in the ocean floor may not be uncommon.

We have no preference between the two possibilities. Further deeper drilling is desirable in order to clarify the origin and formation ages of the Central-Pacific Basin.

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Mercury emissions from chlorine-production solid waste deposits

BEFORE 1970 the amount of Hg discharged to the environment from anthropogenic sources was estimated to be comparable with quantities derived from continental weathering processes¹. As a result, however, of several well-documented cases of acute Hg contamination throughout the world, most large industrial sources have been identified and the discharge of Hg reduced to negligible amounts relative to natural aquatic and atmospheric sources. The natural atmospheric dispersion mechanisms of Hg overshadow input into the global environment by human activity, the latter being of prime importance in localised environments. The role of the chlor-alkali industry in global and regional Hg cycling has been repeatedly discussed²⁻⁵, with the general conclusion that their contribution may be of limited importance globally but of particular concern to local and regional water and air quality⁶.

Although limited studies have been published on losses of Hg from production processes in active chlorine plants^{3,4} none have considered the problem of atmospheric and aquatic emissions from waste products which have been ponded or otherwise

stored near active or inactive plants. The study reported here concerns mercury emissions from wastes stored near an inactive chlorine plant.

The study area is at the site of a large chemical manufacturing facility in the eastern United States which ceased operation in 1972 because of an inability to meet newly imposed state and federal water quality standards. Sodium bicarbonate and related by-products were produced by the ammonia soda process since 1894. The plant began production of chlorine and caustic soda using the Hg-cell process in 1952. Between that time and 1972 waste products from both processes were disposed of in large sludge basins, a procedure relatively common in this industry^{2,4,7}. The disposal site, with a surface area of ~44 ha, is situated adjacent to a river (daily mean discharge = $8.34 \text{ m}^3 \text{ s}^{-1}$) in an area enclosed on one side by a natural ridge and on the river side by dikes built of CaCO_3 waste and boiler ash generated in the NaHCO_3 process.

We collected air, water, and waste samples within the study area during the period 1974–1976. Solid and aqueous samples for Hg determination were handled and analysed according to standard flameless AAS methods⁸. Air samples for particulate and vapour Hg were collected using standard air filter and activated charcoal absorption techniques^{9,10} respectively, and analysed as above.

The volatility of metallic Hg (Hg^0) and many of its compounds suggests its presence in large scale waste deposits such as these to be a potential threat to local air quality. Waste material collected from the surface of the waste ponds was used in laboratory experiments to determine the emission of Hg^0 from a known surface area at controlled temperatures. Approximately 4 kg of waste material (bulk material concentration = $155 \text{ } \mu\text{g Hg g}^{-1}$) was slurried into a 13-litre spherical glass chamber and allowed to dry to a moisture level similar to that observed for the surface of the actual waste ponds (approximately 75% water by weight). Mercury-free air was pumped through the chamber over the waste surface at 1 litre min^{-1} and the filtered output air sampled for Hg^0 . The emission of elemental Hg vapour from the waste material was clearly related to surface temperature (Fig. 1) as expected. The nature of the relationship is similar to that between temperature and the saturation concentration of Hg^0 in air^{2,11} suggesting that the mercury occurs in the waste material to a large extent as

the elemental species. Comparative data on Hg emission rates from natural soils are scarce, although some authors have reported observing release from soils experimentally enriched with inorganic Hg^{12-14} . Natural degassing rates of Hg on regional or global scales have been estimated based on input/output calculations^{15,16} as ranging from 0.02 to $0.03 \text{ } \mu\text{g m}^{-2} \text{ h}^{-1}$, while measured rates for mineralised soils¹⁷ are as high as $1.7 \text{ } \mu\text{g m}^{-2} \text{ h}^{-1}$. We have recently measured the loss of Hg^0 from mercury-contaminated surface soils (total Hg = $99 \text{ } \mu\text{g g}^{-1}$) collected within 1 km of a large active Hg mine, and found emission rates at 33°C to be in the range of 1.4 – $1.7 \text{ } \mu\text{g m}^{-2} \text{ h}^{-1}$. These values are two orders of magnitude lower than those we have measured in comparable conditions for the waste material (120 – $170 \text{ } \mu\text{g m}^{-2} \text{ h}^{-1}$). The difference is probably related to the form of Hg present in the two samples; the mine site soil contains primarily HgS and HgO .

Table 1 Daily mean concentrations of mercury vapour (Hg^0) in air in vicinity of waste ponds under various meteorological conditions

Distance from centre of waste ponds (km)	Time downwind (%) [*]	Mean air temperature ($^\circ \text{C}$)	Concentration of mercury vapour (Hg^0) in air (ng m^{-3})	
			Measured	Predicted
0.4	78	6.3	90	65
0.5	99	6.3	$64 \pm 6^\dagger$	72
1.5	67	6.3	18	26
1.9	21	6.3	12	15
0.5	96	29.4	$991 \pm 2^\dagger$	778

^{*}Expressed as a percentage of the total sampling period (24 h) during which the sample location was downwind of the waste pond.

[†]Replicate samples.

Considering the waste deposits as an area source, we have applied a simple Gaussian diffusion model¹⁸ to estimate 24 h mean concentrations of Hg vapour in the ambient air downwind of the source. The emission rates measured in controlled conditions (Fig. 1) were used in the model; mean wind speed directly over the pond area was assumed to be 1 m s^{-1} ; the background air Hg concentration was taken as 10 ng m^{-3} (ref. 15). Given the simplicity of this approach, the close agreement between measured and predicted 24 h mean air concentrations in similar conditions (Table 1) can be taken as an indication that the entire surface area of the waste pond was emitting Hg vapour at a rate comparable to that measured in controlled experiments.

The levels of Hg^0 in air measured near the pond during periods of low air temperature (Table 1) were slightly elevated above those reported for large US cities^{3,13}. The fact that these concentrations decreased to rural background levels³ within 1.5 km of the waste disposal area is attributable to the ability of a well mixed atmosphere to dilute gaseous pollutants rapidly. However, during periods of higher air temperature, the concentration of Hg^0 measured near the pond area approached the US Environmental Protection Agency suggested standard for total Hg in ambient air ($1,000 \text{ ng m}^{-3}$) (ref. 19). This compares with levels of 30 – $1,600 \text{ ng m}^{-3}$ for air over natural Hg deposits and 10 – $28,000 \text{ ng m}^{-3}$ for natural degassing and geothermal emissions³. The concentration of particulate Hg during this sampling period was surprisingly low (0.25 – 0.36 ng m^{-3}) considering the area in question, and reflected the stability of the waste pond surface to dust resuspension. The indication that essentially 100% of the Hg in air is in vapour form agrees with recently reported results¹⁹.

The location of the waste ponds behind steep dikes along a riverbank and perched on the flank of a ridge is highly conducive to the leaching of soluble components of the waste by direct rainfall and by runoff from the adjacent ridge. Clay tiles installed beneath the dikes serve to drain the waste from the bottom while overflow structures drain the ponds from the surface and intermediate depths. Discharge of waste leachate

Fig. 1 Relationship between emission of vapour phase elemental mercury and surface temperature of waste material. The numerical relationship derived from a nonlinear least squares fit is $Y = a + b \exp(cX)$, where Y = emission rate in $\text{ } \mu\text{g m}^{-2} \text{ h}^{-1}$, X = surface temperature in $^\circ \text{C}$, $a = 6.40$ (standard error = 1.59), $b = 0.310$ (standard error = 0.100), $c = 0.190$ (standard error = 0.00982).

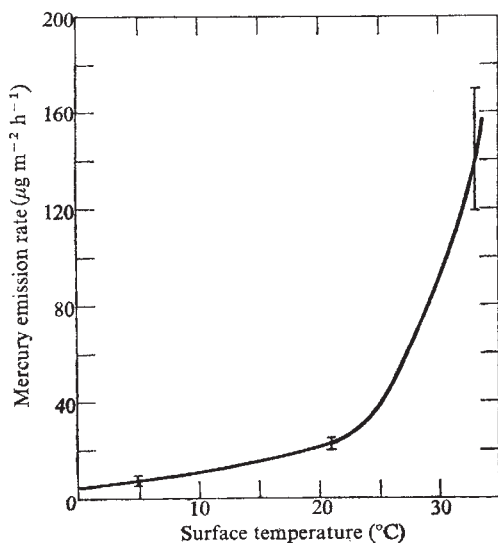


Table 2 Summary of determinations of mercury fluxes from chlor-alkali plant waste disposal pond area using mass balance approach

Upstream*			Downstream†			
Total Hg ($\mu\text{g l}^{-1}$)	Stream discharge‡ ($\text{m}^3 \text{s}^{-1}$)	Total Hg flux (g d^{-1})	Total Hg ($\mu\text{g l}^{-1}$)	Stream discharge‡ ($\text{m}^3 \text{s}^{-1}$)	Total Hg flux (g d^{-1})	Net Hg flux (g d^{-1})
0.077	1.76	11.7	0.220	1.98	37.6	25.9
0.006	1.70	0.88	0.167	1.92	27.7	26.8
0.007	1.61	0.98	0.185	1.81	28.9	27.9
0.008	1.57	1.09	0.193	1.77	29.5	28.4§
0.052	2.83	12.7	0.186	3.19	51.3	38.6
0.012	6.00	6.22	0.116	6.80	68.2	62.0
0.003	11.5	2.98	0.094	12.2	99.1	96.1
0.002	13.1	2.26	0.087	14.8	111	109
0.005	14.5	6.26	0.084	16.3	118	112
0.008	19.1	13.2	0.123	21.5	228	215
0.015	26.7	34.6	0.103	30.1	268	233
0.012	18.2	18.9	0.209	20.6	372	353

*2.7 km above chlor-alkali plant.

†6.4 km below chlor-alkali plant.

‡Based on discharge recorded at nearby US Geological Survey Stream Gaging Station and adjusted for difference in drainage area.

§Measured flux on this day was 14.3 g d^{-1} .

through these structures and many intermittent seepages flow directly into the adjacent river. Our own direct measurements of leaching rates of Hg from the waste area were limited to periods of very low river discharge, since most of the tile drains were submerged and thus inaccessible for sampling and flow measurement during higher flows. Consequently, we also applied a mass balance approach to estimate net fluxes of mercury to the adjacent river (Table 2). Using mercury concentrations and stream discharge measurements taken between 1974 and 1976 at points immediately upstream and downstream of the waste pond area¹⁰, we calculated net fluxes of mercury as the difference between upstream and downstream fluxes. To the extent that mercury accumulates in river bed sediments during periods of low river discharge, or is flushed from river bed sediments during periods of high river discharge, mass balance calculations based on instantaneous measurements will either underestimate (at low discharge) or overestimate (at high discharge) the actual leaching rate of mercury from the waste ponds. Direct measurements of all accessible flows of waste leachate into the adjacent river during a period of low river discharge offered some reasonable verification of a mass balance calculation made for the same period. On one day the total measured flux of mercury was 14.3 g d^{-1} while the calculated net flux was 28.4 g d^{-1} . The lower measured flux probably results from our inability to locate and measure all leachate seepages rather than from an overestimate by the mass balance calculation.

Our estimates of aquatic fluxes of mercury from the waste pond area indicate considerable variability in the apparent leaching rate of mercury from the waste, but demonstrate a good correlation ($r = 0.86$; $P < 0.01$) with discharge of the adjacent river which responds primarily to local rainfall in the upstream 575 km^2 catchment. However, it is not clear whether this correlation results from a direct dependence of mercury leaching rate on rainfall input or from the dependence of bed sediment erosion on river discharge. Both processes probably contribute to higher net mercury fluxes from the waste pond area during periods of higher river discharge.

Using simple regression analyses we have obtained functions which estimate both surface emission of Hg^0 , based on air temperature, and aquatic leaching of Hg, based on streamflow. Applying these relationships to daily streamflow and hourly temperature data for the waste pond area for a typical year (October 1973–September 1974), we have calculated the estimated annual loss of mercury from the waste disposal site by both the atmospheric and aquatic pathways. Losses were nearly equally divided between these pathways $36 \pm 4 \text{ kg yr}^{-1}$ (predicted sum $\pm$ the estimated standard error of the sum of predicted fluxes) atmospheric emission of Hg and $39 \pm 2 \text{ kg yr}^{-1}$

aquatic emission of Hg. The occurrence of high streamflow during cold winter months resulted in predominance of the aquatic pathways during winter and predominance of the atmospheric route during summer months. This results in a relatively uniform emission of Hg from the waste deposits over an annual cycle.

The estimated total annual flux of Hg from this inactive plant, $75 \pm 5 \text{ kg yr}^{-1}$, is approximately 6% of the US Environmental Protection Agency standard for emission of Hg to air and water from active chlorine plants situated in riverine environments. Recently reported losses of Hg to the atmosphere and surface waters by Swedish chlor-alkali plants were, however, in the range of $150\text{--}1,400 \text{ kg yr}^{-1}$ (refs 2, 6). Values for US plants are estimated to range up to $1,100 \text{ kg yr}^{-1}$ (ref. 3). Estimates of the capability of improved technology to reduce Hg emissions from active plants indicate a possible 40% decrease in emissions by these routes³, resulting in Hg losses from the more efficient operations (that is, those reported above for Swedish plants) which would be similar to those reported here for waste disposal ponds alone.

At present the most significant mercury waste problem in this industry involves disposal of large quantities of solid waste materials. Thus, given the common disposal practice of ponding solid wastes, along with the residence time of Hg in existing large scale waste deposits (conservatively estimated as 100 years for the waste area studied here)¹⁰, it becomes apparent that the role of such residual waste deposits in local Hg cycles will continue to grow in relative importance.

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Dissolved aluminium concentration in sea water

DISSOLUTION of clay minerals in seawater results in aluminium concentrations in solution (1.3–7.5 $\mu\text{g Al per l}$) which are similar to those found in coastal waters but considerably higher than the probable value of the aluminium concentration in open ocean surface waters (0.5 $\mu\text{g Al per l}$). This note compares the results of precipitation and dissolution and investigates the mechanism of the dissolution of aluminium from three different clay minerals. Our results show that bottom and suspended clay sediments probably act as a source of dissolved aluminium to sea water, whereas removal below clay solubility predictions is probably the result of biological activity, as is the case for silicon, the other main constituent of most rocks and minerals.

Recent results from the North Sea¹ and the North Atlantic² show that away from the influences of aluminium-rich freshwater sources and higher coastal concentrations of suspended matter the concentration of aluminium in seawater will be approximately 0.5 $\mu\text{g l}^{-1}$. This level is more than an order of magnitude lower than that predicted by alumina-silica coprecipitation experiments³, which showed that at levels of silica which occur naturally in seawater, the solubility of aluminium is much reduced below that which is stable in silica free seawater. These experiments³ may have produced erroneously high levels due to the inclusion of particulate aluminium in the analysis results and the failure of the experiments to reach complete equilibrium.

We carried out the following experiments (a fluorometric analysis method was used which is insensitive to aluminium in particulate matter⁴). The fractions of the clays of less than 2 μm were separated by centrifugation, and suspensions containing 10 g of clay in 1 l of sea water were prepared in polythene bottles. The pH of the suspensions were adjusted to pH 8.1 with a few drops of sodium carbonate solution. They were then maintained at 20 °C in the dark and continually shaken for four months. Aliquots were withdrawn periodically, centrifuged and filtered through 0.1- μm membrane filters before the aluminium and silicate analyses were carried out. The

clays and seawater reached a steady state within three months. No trends are discernible in the measurements made at three and four months. After four months the pH of the solutions were readjusted to pH 8.1 using dilute sodium hydroxide, and maintained at this pH by daily control for one week before the aluminium and silicate concentrations were measured. The conditions were again changed by lowering the temperature to 3 °C and the new conditions were maintained for one week before the analyses were carried out. The results of this experiment are summarised in Table 1.

The pattern of silica dissolution followed that observed in previous studies⁵: a rapid initial rise in the concentration was followed by a more gradual increase over several weeks. The change in aluminium followed a completely different path after the initial sharp increase. For each of the three clays the highest aluminium concentrations were measured in the first aliquot to be withdrawn. The concentrations then fell gradually reaching apparent equilibrium in two months. To see to what extent the fall in aluminium concentrations observed was due to changes in pH, a second experiment was carried out using kaolinite, the pH being maintained at pH 8.1. The results of this experiment are shown in Fig. 1. The decrease in aluminium concentrations observed in the first experiment were not simply due to pH changes. Also, there was no evidence for a simple control of the aluminium concentration by silicate as the aluminium concentration continued to decrease after the maximum silicate concentration was reached. The plot of aluminium concentration against $t^{1/2}$ is approximately linear which suggests the rate controlling step is a diffusion process, which is amenable to further study.

When the results of these experiments (1.3–7.5 $\mu\text{g Al per l}$) are compared with those for precipitation (8–15 $\mu\text{g Al per l}$) (ref. 3) we see that dissolution, as would be expected for approaching equilibrium from the opposite direction, gives slightly lower values than precipitation. Both sets of results predict much higher concentrations of aluminium in seawater containing an average amount of silica than have actually been observed in open seawaters. In coastal waters aluminium concentrations of the predicted magnitude have been recorded (maximum levels observed in refs 1 and 2 are 5.5 and 4.6 $\mu\text{g Al per l}$ respectively). The inference from comparing the results for open seawater and coastal seawater is that away from the higher suspended matter concentrations in coastal waters, the clay concentration is not sufficiently great to maintain the aluminium concentration in competition with biological activity which is removing aluminium from the water. A number of pieces of evidence support the idea of aluminium being removed from solution in some part of the life cycle of siliceous diatoms. Diatom frustules are known to contain aluminium⁶, the source of which is uncertain. Possible montmorillonite structures have been observed in the frustules from living diatoms thought to be uncontaminated by detrital clays⁷. This suggests that aluminium

Table 1 Summary of results of first clay* dissolution experiment

Result†	pH	T (°C)	Kaolinite		Fireclay		Montmorillonite	
			Al ($\mu\text{g l}^{-1}$)	SiO ₂ (mg l ⁻¹)	Al ($\mu\text{g l}^{-1}$)	SiO ₂ (mg l ⁻¹)	Al ($\mu\text{g l}^{-1}$)	SiO ₂ (mg l ⁻¹)
(1)	8.1	20	13.8	3.8	12.5	2.8	5.4	5.4
(2)	—	20	2.7	5.6	6.0	3.2	1.3	12.2
(3)	—	20	2.7	5.6	5.9	3.2	1.3	12.4
(4)	8.1	20	7.3	4.3	7.5	2.6	4.8	6.8
(5)	8.1	3	3.1	3.5	5.3	2.1	3.0	5.3

*Clays used: Kaolinite (A.P.I. H7), Fireclay (Glashafenton 1), Montmorillonite (Tixoton). Seawater collected 26 Jan. 1976 from N. Atlantic 59°4'N, 02°50'W. Initial concentrations: Aluminium 0.3 $\mu\text{g Al per l}$, Silica 0.1 mg SiO₂ per l.

†Results: (1) after 36 h; (2) after 3 months; (3) end of 4 months, final pHs—Kaolinite 7.35, Fireclay 7.69, Montmorillonite 7.35; (4) after 4 months plus 1 week at pH 8.1; (5) after 4 months plus 1 week at pH 8.1 and 1 week at pH 8.1 and 3 °C.

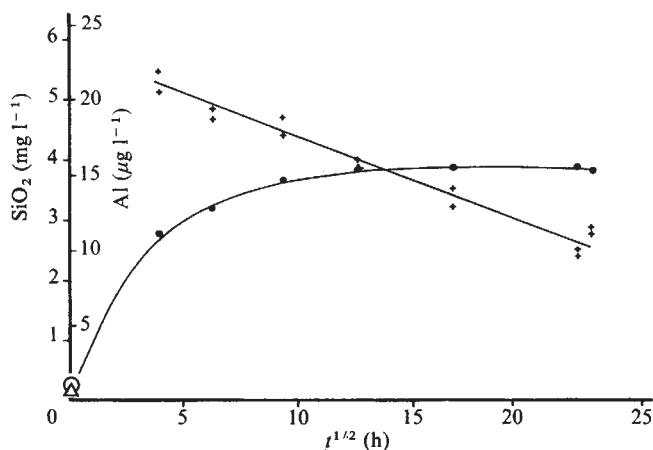


Fig. 1 Rate of dissolution of silica (●) and aluminium (+) from duplicate suspensions of kaolinite, $10,000 \text{ mg l}^{-1}$ in sea water at pH 8.1 and $19 \pm 1^\circ \text{C}$. For silica the difference between the samples taken is smaller than the data point. Initial concentrations of: ○, silica $0.1 \text{ mg SiO}_2 \text{ l}^{-1}$; △, aluminium $0.3 \mu\text{g Al l}^{-1}$.

removal may be an active biological process as the removal from biologically productive waters seems to be for other trace metals^{8,9}. However, evidence also exists for the formation by adsorption on to the frustules of dead diatoms of aluminosilicate phases¹⁰. Further work is required to establish how strongly aluminium is adsorbed by siliceous diatom remains and what the balance is between that and active biological removal.

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Optimal foraging in great tits (*Parus major*)

OPTIMAL foraging theory^{1–5} is based on the supposition that animals have been designed, by natural selection, to behave in a way that will maximise their inclusive fitness and that in terms of foraging this goal may be approximated by maximising the net rate of energy intake while feeding^{1–4}. Therefore, given the constraints of any particular situation it is possible to predict how an animal ought to behave while foraging. I have tested an optimal foraging model which predicts how a predator should exploit patchily distributed prey. The results are consistent with the predictions of the model.

The prey of most predators are not scattered randomly throughout the environment⁶ but occur in aggregations, which I call patches. To exploit these patches efficiently a predator needs to 'decide' which patches to visit and when to move from one to another. If the quality of a patch cannot be predicted in advance the problem simply becomes: how long is it best to

remain in each individual patch? Several authors have produced similar models describing the optimal solution to this problem^{7–9}. Owing to depletion of prey, the capture rate within a patch is likely to be a decreasing function of the time spent there¹⁰. This means that if a predator stays for too long its capture rate will fall below that which could be achieved by moving to the next patch. Conversely, if it does not stay for long enough it will spend too large a proportion of its time travelling. The optimal solution is that the predator should move when its capture rate within a patch drops to a threshold equal to the average capture rate for the environment as a whole. This critical threshold is called the marginal capture rate⁷ (Fig. 1).

The optimal solution ($Tp(\text{opt})$) is affected by changes in either the curve $f(Tp)$ or the time taken to travel between patches (Tt). In my experiments I manipulated the latter and kept the former constant so that it is possible to define the relationship between $Tp(\text{opt})$ and Tt as follows.

Assuming the curve $f(Tp)$ is an exponential of the form

$$f = Q[1 - \exp(-mTp)] \quad (1)$$

(where f is average net energy intake; Tp is time in patch; m and Q are constants), the rate of food intake after a length of stay Tp is given by

$$df/dTp = Qm \exp(-mTp) \quad (2)$$

The optimal predator should leave a patch when its capture rate falls to the average capture rate for the environment^{7,8}. That is when

$$df/dTp = f/(Tt + Tp(\text{opt}))$$

where Tt is mean travel time.

Therefore

$$Qm \exp(-mTp(\text{opt})) = f/(Tt + Tp(\text{opt})) \quad (3)$$

Substituting equation (1)

$$Qm \exp(-mTp(\text{opt})) = Q[1 - \exp(-mTp(\text{opt}))]/(Tt + Tp(\text{opt})) \quad (4)$$

Rearranging this gives

$$Tt = 1/[m(\exp(mTp(\text{opt}))) - 1] - Tp(\text{opt}) \quad (5)$$

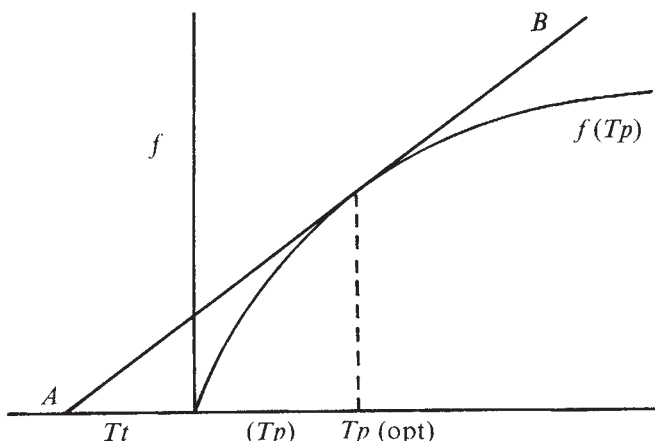


Fig. 1 Graphical representation of a model of optimal patch use^{7,8}. The curve $f(Tp)$ represents the average net energy gain (f) as a function of the time spent in each patch (Tp). The abscissa is extended in the negative quadrant to a distance Tt which is the mean time taken to travel between patches. The maximum average net rate of food intake that is obtainable can be determined by constructing the tangent to $f(Tp)$ from point A. The predator ought to leave each patch after time $Tp(\text{opt})$ in order to maximise its average capture rate.

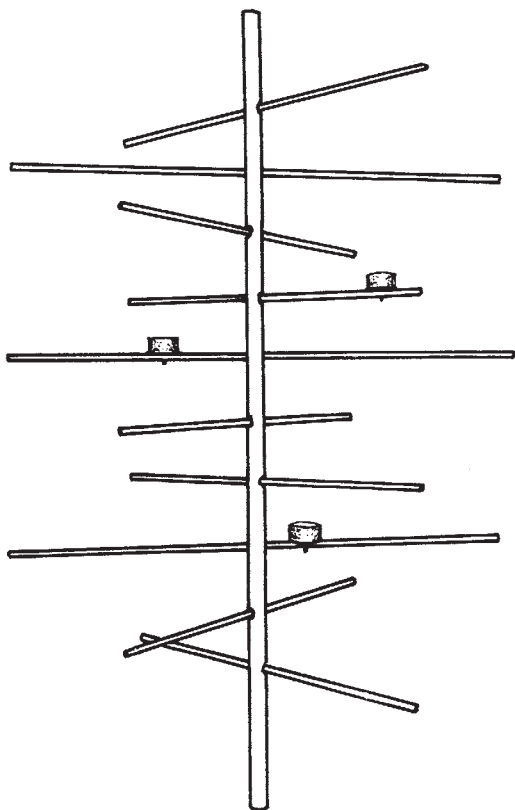


Fig. 2 An experimental 'tree'.

This relationship makes it possible to test the predictions of the model quantitatively.

I performed the experiments on six wild caught great tits (*Parus major*). The apparatus consisted of an aviary (4.6 m × 3.7 m), containing five artificial trees (Fig. 2). Each tree supported six patches. Patches were made from 4-cm lengths of plastic drainpipe (6.5 cm diameter), which were sealed at one end. The patches were filled with sawdust in which I hid the prey, six quarter sections of mealworm.

I subjected each bird to two types of 'environment' which were identical in all respects except for the time and energy cost of travelling between patches. Ideally these costs should have been increased by placing the patches farther apart but it was impossible to separate them by large enough distances within the confines of the aviary. Instead, I covered each patch with a cardboard lid which the bird had to remove before it began foraging. There were two types of lid. One rested on top of the drainpipe and could easily be flipped off, the other fitted just inside the rim and had to be prised out. Each experimental environment contained patches with lids that were either all 'hard' or all 'easy' to remove, these differences corresponding to long and short travel times respectively.

I performed six trials on each bird in each environment. Every trial was preceded by 2 h of food deprivation and was terminated when the bird had foraged for 10 min. Satiation and overall depletion of the environment had no observable effect within this period. Throughout each trial I watched the bird from behind a one-way mirror and recorded its behaviour on a Dawkins' behaviour organ¹¹.

Table 1 shows that the changes in travel time have a significant effect on the average capture rate and the average time spent in each patch. To calculate $Tp(\text{opt})$ from equation (5) it is necessary to know the formula for the curve $f(Tp)$ (Fig. 1). I determined this curve by calculating the mean number of prey caught as a function of the time spent searching in a patch. For each bird the best fit was provided by an exponential of the form described previously (equation (1)). Figure 3 shows the actual relationship between the mean travel time and the mean time spent per patch, for each bird in both environments. The dotted

Table 1 Summary of principal variables in each environment

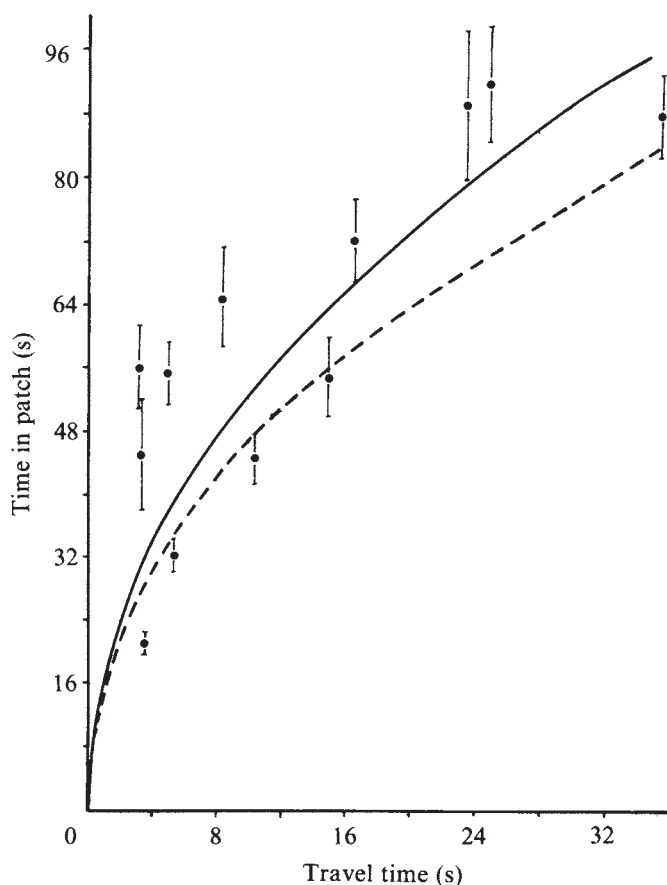
Variable	'Hard' environment	'Easy' environment	Paired <i>t</i> value	Significance level, 5 d.f.
Mean intercatch interval (s)	15.37 (1.37)	14.41 (1.87)	0.98	NS
Time in patch (s)	73.67 (8.06)	45.80 (6.77)	2.95	2-tailed $P < 0.02$
Handling time (s)	6.79 (0.60)	9.62 (1.65)	1.51	NS
Travel time (s)	21.03 (3.65)	4.76 (0.80)	4.73	2-tailed $P < 0.002$
Average capture rate (prey per s)	0.0264 (0.00277)	0.0366 (0.00541)	3.52	1-tailed $P < 0.02$

Averages of data for all six birds. The figures in brackets are standard errors. The average capture rate was determined by dividing the total number of finds in each trial by the total foraging time. The mean intercatch interval within a patch is the average time spent searching for each prey item. Handling time is the time taken to manipulate and eat a prey item. Time in patch and travel time are explained in the text.

curve is the optimal solution calculated using equation (5) and the mean $f(Tp)$ curve for all six birds. Most of the data points lie above this curve which indicates that the birds tended to stay in each patch for too long. This can be explained by the fact that the curve $f(Tp)$ only takes into account the average gross rate of energy intake. If adjustments are made for the energetic costs of travelling and searching the relationship in equation (5) can be rewritten as

$$Tt = [KQ(1 - \exp(-mTp))(1 + mTp)] - P/(KQm \exp(-mTp) - B)$$

Fig. 3 The relationship between the mean travel time and the mean time spent in each patch, for each bird in both experimental treatments. The vertical bars represent the standard error. The dotted curve shows the optimal solution derived from equation (5) and the unbroken curve shows the optimal solution after making adjustments for energy expenditure. $P = 0.697 \text{ cal s}^{-1}$; $B = 0.155 \text{ cal s}^{-1}$; $k = 56 \text{ cal}$; $Q = 6.3587$; $m = 0.00813576$.



where P is energy cost of travel (cal s^{-1}), B is energy cost of searching (cal s^{-1}), K is calorific content of prey (cal). The basal metabolic rate (BMR) of a great tit was calculated using the equation of Aschoff and Pohl¹². Estimates of the energy requirement for flight and feeding were taken as $9 \times \text{BMR}$ and $2 \times \text{BMR}$ respectively^{13,14}. The solid curve in Fig. 3 shows the optimal solution based on net energy intake. It is not significantly different from the observed data. (Paired comparisons $t = 1.65$ ($t_{0.05} = 2.20$)).

These results show that great tits can exploit patchily distributed prey in a manner predicted by optimal foraging theory. Previous attempts to test this type of model^{15,16} have been based on qualitative predictions, whereas I tested a quantitative relationship. The results imply that the birds can estimate both their instantaneous rate of intake within a patch and the average rate of energy intake over their environment as a whole. Unfortunately this concept of 'environment' is ill defined and it is difficult to know over what sort of time or area the birds might be averaging. One possible solution is that they have a memory window which produces a sliding average over the last few patches or minutes. In a constant environment a large window would result in an accurate measure of the average capture rate. In a fluctuating environment, however, a very large window could result in the predator adapting too slowly to changes in prey density. It seems likely that there is an optimal size for this memory window, and experiments to determine its size in great tits are underway.

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Nature and precursors of juvenile hormone-induced excessive cuticular melanisation in German cockroach

GROWTH in insects proceeds through a number of moults, when the old cuticle is shed and a new one synthesised. Within few hours of ecdysis, the cuticle hardens and develops the characteristic colour pattern. When we treated German cockroach *Blattella germanica* (L.) nymphs with insect juvenile hormone (JH) 12 h before ecdysis, the resulting nymphs or adults developed excessive black coloration all over their bodies, obliterating the normal colour patterns¹. The nature of this visible change was unknown^{2,3}. Here we show for the first time that the excessive coloration, following JH treatment, is caused by melanin, and that dopamine or catechol seems to be involved in the hormone-influenced pathway.

The striped pattern of the dorsal plate (pronotum, Fig. 1) provided ready identification of melanised cockroaches. When a melanised nymph moulted into an older nymph or adult, the abnormal coloration was lost together with the old cuticle. Since only the outer layers of the integument are shed at the moult, this meant that the dark pigment was contained in these outer layers and not in the underlying

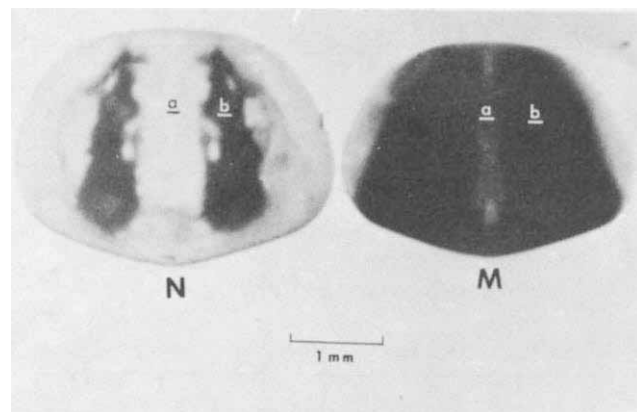
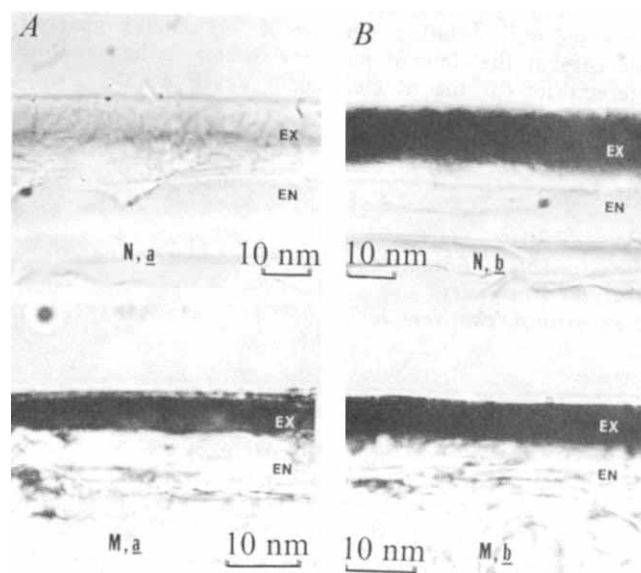


Fig. 1 Pronota of normal (N) and melanised (M) *Blattella germanica* adults showing the non-melanised (a) and melanised (striped, b) zones in normal adult and the corresponding zones in melanised adult. Synthetic *Cecropia* juvenile hormone, methyl-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate, (or any of its mimics⁸) was applied topically (1.5 μl), by injection (0.33 μl), or by treating the food (200 p.p.m.).

epidermis. Cross sections of the cuticles (Fig. 2) confirmed that the pigment was located in the exocuticle, and that it was present even in areas that are normally devoid of pigment. We isolated the black pigments from the normal and melanised cuticles and studied their infrared spectra in comparison with that of synthetic melanin (Fig. 3). We found the cuticular black pigments to be quite comparable with the synthetic melanin and that the spectra from normal and melanised cuticles were identical. We therefore determined the amounts of melanin in the normal and melanised cuticles fluorimetrically⁴, and found that the melanised cuticles contained 6.5 mg melanin (per g dry cuticle) compared with 4.8 g in the normal cuticles—a 35% increase.

We then proceeded to identify the precursors that might be on the pathway to excessive melanin biosynthesis. We

Fig. 2 Cross section of pronota of normal (N) and melanised (M) *Blattella germanica* adults; 10 μm , unstained. The clear zones (A) and striped zones (B) of the normal and melanised pronota are compared. Note the presence of melanin in zone a of melanised cuticle obliterating the normal melanin pattern. EN, Endocuticle; EX, exocuticle.



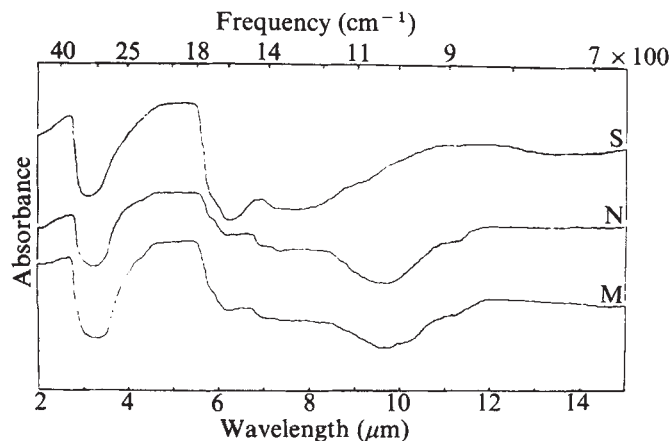


Fig. 3 Infrared spectra of synthetic melanin (S) and cuticular melamins of normal (N) and melanised (M) *Blattella germanica* adults using KBr disks containing 1% melanin. Dried cuticles were hydrolysed in acid and melanin was extracted in alkali⁹⁻¹².

incubated the fresh, soft, untanned cuticle (with the underlying epithelium intact) from normal cockroach in a tissue culture medium⁹ modified by deleting tyrosine, tryptophan, phenylalanine, and foetal calf serum. Treated media were prepared by selectively incorporating the substrates and/or inhibitors into the culture medium—all at a final concentration of 3×10^{-3} M and pH 7.0. The media were oxygenated for 5 min through a fine capillary tube before introducing the cuticle. After incubating in the media for 5–10 h at 35 °C, the cuticles were washed in 95% ethanol and the coloration observed. The presence of tyrosine alone was adequate for the development of normal colour pattern. This could be inhibited, however, by either diethyldithiocarbamate (an inhibitor of tyrosinase⁶, EC 1.14.18.1) or 1-(DL-erythyl)-2-(2,3,4-trihydroxybenzyl) hydrazine HCl (an inhibitor of dopadecarboxylase⁷, EC 4.1.1.28), showing that the cuticle normally contains all the required enzymes for melanin biosynthesis. Also, there was no need for JH in the medium. If either dopamine or catechol was provided in lieu of tyrosine, there was excessive melanisation similar to that observed in the JH-treated individuals. It is possible that the exogenous JH interferes with the timely auto-inhibition of tyrosinase *in vivo* leading to unrestricted melanisation. Alternatively, a more readily utilisable precursor like dopamine is made available to the integument by induced synthesis, activation of stored molecules, or increased transportation. Absence of JH, which is generally the case at the time of moulting, seems to be a natural prerequisite for the normal colour development.

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Simultaneous recording from several neurones in an invertebrate central nervous system

THE ability to monitor activity in many neurones simultaneously might provide a powerful tool for solving certain problems in neurobiology. There are several changes in the optical properties of a neurone that occur during an action potential¹⁻³ that might be used in an apparatus that could monitor spike activity in many neurones simultaneously. Although the changes in intrinsic (native) optical properties have been useful in other instances where an optical measurement of membrane potential was desired, larger signals were needed for this application. Therefore, a search was begun for extrinsic probe molecules which could serve as sensitive molecular transducers in stained membranes, transforming changes in membrane potential into changes in light intensity^{4,5}. In earlier experiments we demonstrated changes in fluorescence⁶ and absorption⁷ during action potentials in individual neurones of a segmental ganglion from the leech, *Hirudo medicinalis*. In those experiments the ganglion was stained with a merocyanine dye⁸. In the experiments described here, carried out on the supraoesophageal ganglion of the barnacle, *Balanus nubilus*, the absorption change of a new merocyanine-oxazolone dye^{9,10} was used. In measurements on squid giant axons, the signal obtained with the new dye was five times larger. In addition, the photodynamic

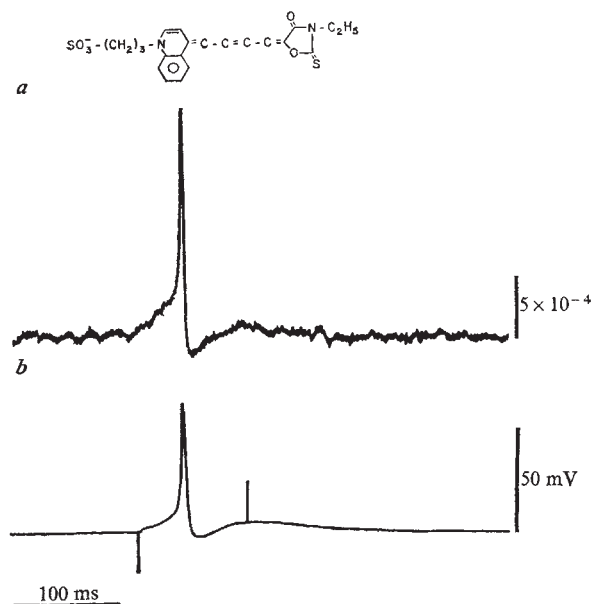


Fig. 1 The change in absorption (a) during an action potential (b). The structure of the merocyanine-oxazolone dye used in these experiments is shown at the top. The action potential size and time course were unaffected by turning on the light; no photodynamic damage was detected in the barnacle experiments even though the saline was equilibrated with air. The direction of the arrows to the right of the optical traces indicates an increase in absorption and the length of the arrow represents the stated value of the change in absorption divided by the resting absorption. An interference filter with peak transmission at 720 nm and width at half-height of 30 nm was positioned between the light source and condenser lens to restrict the measurement to wavelengths where the signal was largest in squid experiments. The output of each photodetector was amplified, limited to a band-pass of 1 Hz–0.3 kHz, and then displayed on a storage oscilloscope; permanent records were made with a Polaroid camera. The records were traced from enlargements of the original photographs. The ganglia were pre-incubated with a 1.5 mg ml⁻¹ solution of collagenase in barnacle saline¹² for 5 min. The experiments were carried out at room temperature, 20–23 °C.

damage^{5,11} associated with photochemical effects of intense illumination in the presence of dye and oxygen was 100 times smaller with the new dye.

To measure the absorption of light by individual neurones, an enlarged image of the ganglion was formed with a $\times 20$, 0.4 n.a. objective using a Leitz Ortholux II microscope. Light guides (Schott, LST, clad glass rod) were used to carry the light from images of the individual neurones to individual photodiodes (PV 100A, E.G. & G. Inc.). The light guides and photodiodes were fixed to magnets on a stainless steel annulus above the image plane. The tips of the light guides had diameters of 1.5 mm and were positioned over the images of neurones by moving the magnets. The sizes of the large neurones on the dorsal surface of the ganglion were between 40 and 90 μm .

The barnacle ganglia were incubated for 20 min with a 1 mg ml^{-1} solution of the merocyanine-oxazolone dye shown in Fig. 1 (available as NK 2367 from Nippon Kankoh-Shikiso Kenkyusho, Okayama, Japan). The dye solution was then replaced with saline and an individual neurone impaled with a microelectrode. The tip of a light guide was positioned over its image, and a depolarising current was then passed through the microelectrode to stimulate the neurone. A simultaneous optical and electrode measurement is shown in Fig. 1. The change in intensity at the peak of the action potential divided by the resting light level was only about 10^{-3} and was often smaller. Even so, the signal-to-noise ratio in Fig. 1 is greater than 10:1. But, it was only after considerable effort¹³ that we were able to reduce the noise from mechanical vibrations and other optical disturbances so that most of the noise in Figs 1 and 2 results from random arrival of photons at the photodetectors (shot noise¹⁴). Significant improvements in the signal-to-noise ratio can be achieved only by modifying the apparatus to increase the intensity reaching the photodetectors or by finding more sensitive dyes.

The signal-to-noise ratio illustrated in Fig. 1 was large enough so further experiments were carried out to determine whether synaptic potentials could also be detected optically. In neurones with excitatory or inhibitory synaptic potentials of 10 mV or greater, we could reliably detect the synaptic events in the optical trace. In the best circumstances, synaptic potentials of only 4 mV could be detected optically¹⁵.

In the same experiment as that shown in Fig. 1, seven additional light-guide-photodiodes were positioned over the images of seven other neurones so that the activity of eight neurones was monitored simultaneously. In addition to the action potential in the impaled cell (7), there were spontaneous action potentials in two other neurones (1, and 4). We are confident that the absence of spikes in the remaining traces results from the absence of action potentials in those cells. (In several experiments, when a nerve root was stimulated with a suction electrode, optical spikes, presumably representing antidromic action potentials, appeared in previously silent neurones. These experiments are reported more fully in ref. 13.)

The magnitude of the optical signal will depend on several factors including membrane area and the amount and site(s) of dye binding in addition to the magnitude of the change in membrane potential. Since it is difficult to evaluate each of these parameters, it is not possible at present to determine the magnitude of the potential changes from these optical measurements; only information about the time course and the direction of the potential changes is obtained. Neither pharmacological effects nor photodynamic damage due to the merocyanine-oxazolone dye were detected in experiments on barnacle ganglia. Our evaluation of such effects is preliminary, however.

We think that the apparatus used in the experiment shown in Fig. 2 can be expanded so that 20 neurones are monitored simultaneously. It may be possible, using an

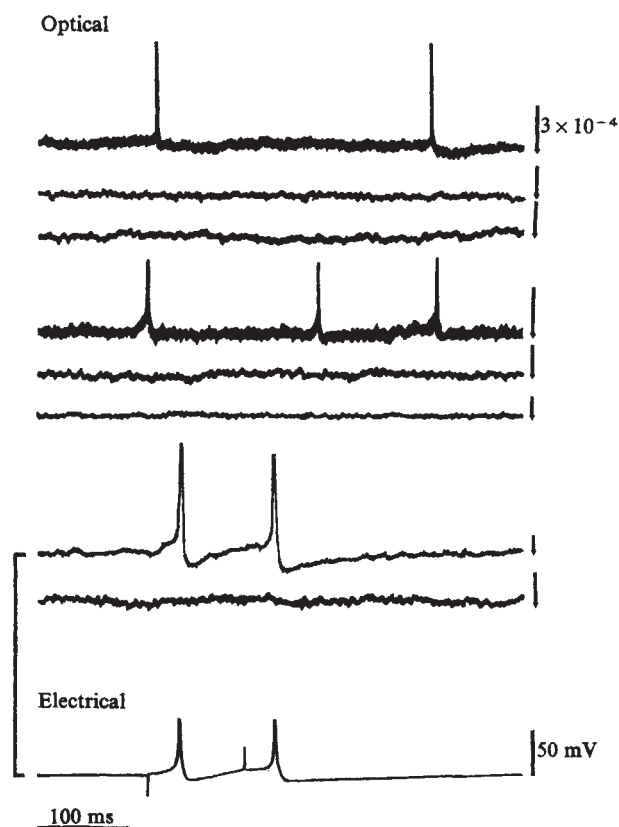


Fig. 2 Simultaneous optical recording from eight neurones. Neurone 7 was stimulated by passing current through the microelectrode; spontaneous action potentials are recorded from neurones 1 and 4. Each arrow represents a $\Delta A/A$ of 3×10^{-4} . ($\Delta I/I$, the observed fractional change in light intensity reaching the photodetector, is about half as much.)

electronic image detector positioned at the objective image plane, to develop an apparatus that monitors activity in 50–100 neurones. The ability to monitor activity from many cells may prove valuable in helping to determine the functional connections between neurones in nervous systems with a small number of neurones. Experiments designed to determine the neuronal basis of very simple behaviours might also be facilitated.

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Antagonism of ethanol narcosis by histidyl-proline diketopiperazine

THYROTROPIN-RELEASING hormone, pyroglutamyl-histidyl-prolineamide (TRH, Fig. 1) belongs to the class of hypothalamic peptides termed releasing factors¹. Although TRH was originally described as the hormone which stimulates the release of thyrotropin, it has subsequently been shown

to be involved in many other biological processes¹. The mechanism by which TRH exhibits this broad range of activities is not clear; it might involve the action of TRH at multiple recognition sites or TRH might serve as a precursor for compounds which are biologically active. Previous studies on TRH metabolism have demonstrated the formation of pyroglutamyl-histidyl-proline (acid TRH, Fig. 1)¹. Attempts to associate biological activity with this compound have been uniformly negative, providing no support for the precursor hypothesis^{2,3}. We show here that, in addition to acid TRH, TRH is also metabolised to histidyl-proline diketopiperazine (His-Pro, Fig. 1)^{4,5}. This cyclic dipeptide is substantially more potent than TRH in reducing ethanol-induced sleep in rats⁶. These data indicate that TRH metabolism plays a part in its biological effects.

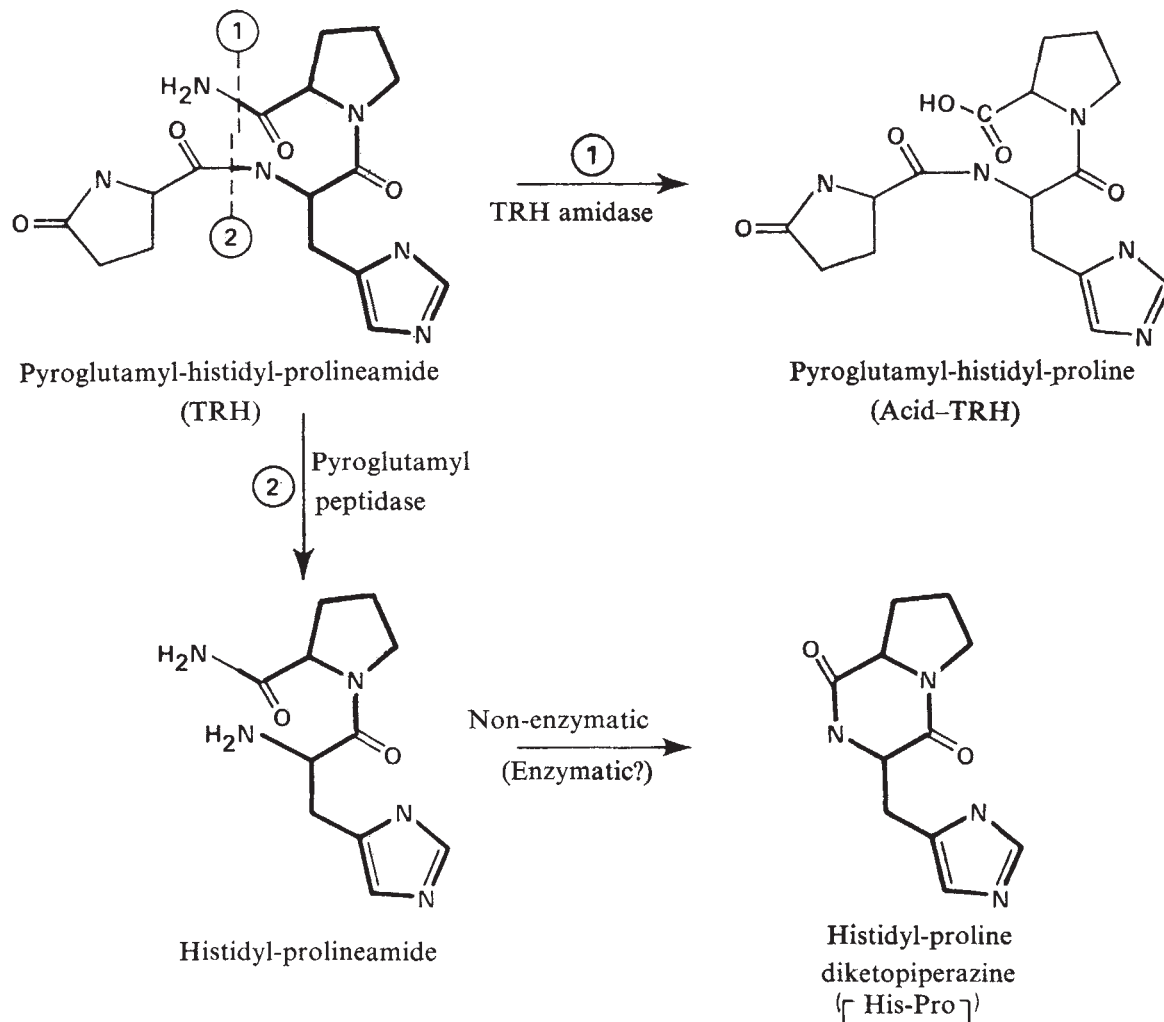


Fig. 1 Pathway of TRH metabolism in rat brain. The portion of the structure of TRH designated by the heavy lines corresponds to the region which gives rise to histidyl-prolineamide and histidyl-proline diketopiperazine. Synthetic L-histidyl-L-proline diketopiperazine was prepared by coupling benzyloxycarbonyl-L-histidine (20 mmol, ICN) with L-prolineamide (20 mmol, Vega-Fox) in the presence of hydroxybenzotriazole (40 mmol, Aldrich), N-ethylmorpholine (20 mmol, Aldrich) and dicyclohexylcarbodiimide (20 mmol, Pierce). The solution in 175 ml of dimethylformamide was stirred in an ice-bath for 3 h and then overnight at room temperature. After removal of the precipitated dicyclohexylurea, the solution was concentrated to dryness *in vacuo*, then dissolved in approximately 25 ml of H₂O. The solution was applied to a column (3 × 20 cm) of diethylaminoethylcellulose (Whatman DE-23, free base) previously equilibrated with H₂O and eluted with H₂O (10-ml fractions). Those fractions which were Pauly positive¹⁰ were pooled and concentrated to dryness *in vacuo*. The intermediate benzyloxycarbonyl-L-histidyl-L-prolineamide was crystallised four times from hot H₂O (yield approximately 500 mg; microanalysis for C₁₈H₂₂N₄O₅ (Microanalysis, Inc., Wilmington, Delaware), showed (in %): 58.94, 6.04, 18.04, 16.90). The calculated values are: 59.21, 6.02, 18.17, 16.60. Benzyloxycarbonyl-L-histidyl-L-prolineamide (500 mg) was dissolved in a solution containing methanol (10 ml), H₂O (0.3 ml) and glacial acetic acid (0.3 ml). After adding palladium black catalyst (100 mg), the suspension was hydrogenated under 40 pounds pressure for 2 h. The catalyst was removed by filtration and the resultant solution was heated in a boiling H₂O bath for 30 min, then dried *in vacuo*. After dissolving the product in H₂O, it was purified by passage through a column (2 × 5 cm) of DEAE-cellulose; the column was washed with H₂O. The Pauly-positive fractions were concentrated to dryness *in vacuo* to yield crystalline L-histidyl-L-proline diketopiperazine (yield approximately 200 mg). Microanalysis for C₁₀H₁₂N₄O showed (in %): 54.75, 6.08, 22.10, 17.22. The calculated values for His-Pro · 0.6 H₂O are: 53.91, 6.25, 22.86, 16.97. Mass spectrographic analysis showed prominent ions at *m/e* 234 (the molecular ion of His-Pro), *m/e* 154 (the proline-glycine diketopiperazine ion), *m/e* 81 (the methyl imidazole ion) and *m/e* 70 (the pyrroline ion). ¹H-magnetic resonance spectral analysis at 100 MHz showed chemical shifts at 1.95, 2.2, 3.4, 3.5, 4.65, 7.4 and 8.25 p.p.m. downfield from trimethylsilane. Amino acid analysis of an acid hydrolysate (6 M HCl, 15 pounds inch⁻², 180 min) showed the presence of ninhydrin-positive material corresponding only to proline and histidine (ratio 1.1:1).

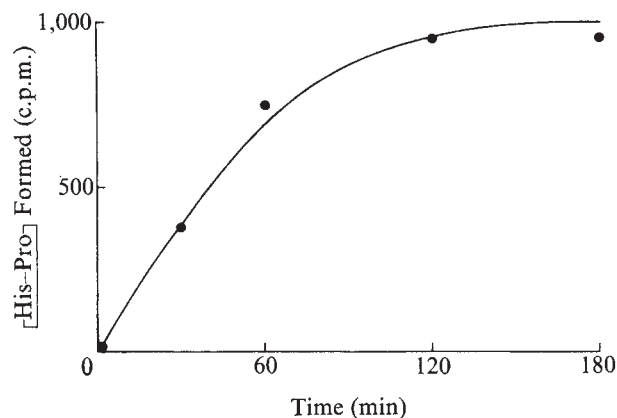


Fig. 2 Non-enzymatic formation of His-Pro from His-ProNH₂. [³H-Pro]-His-ProNH₂ was prepared from [³H-Pro]-TRH (New England Nuclear) as follows: 41 μmol of [³H-Pro]-TRH (1.21 μCi μmol⁻¹) was incubated with 50 μmol Tris-HCl (pH 7.5) and 800 μl *Aerobacter cloacae* pGlu-peptidase (purified up to the Sephadex G-100 stage as described by Doolittle and Armentrout⁷, activity = 0.3 μmol pGlu-Ala hydrolysed min⁻¹ ml⁻¹) for 3 h at 25 °C in a total volume of 1 ml. At the end of the incubation period, the whole reaction mixture was applied to a sheet of Whatman 3 MM (60 × 45 cm) paper and chromatographed in 1-butanol-acetic acid-H₂O (4:1:1) (ascending, overnight, 25 °C). The area on the paper corresponding to His-ProNH₂ (*R_f* = 0.12–0.13, 4.7% of the total radioactivity) was cut out and extracted with 10 ml of 90% methanol. The methanol extract was filtered and concentrated *in vacuo* at 25 °C. The [³H-Pro]-His-ProNH₂ comigrated with authentic His-ProNH₂ on paper chromatography (ascending, 25 °C, 15 h, Whatman 3 MM, chloroform-methanol-H₂O (5:5:1), (Solvent 1) *R_f* = 0.42) and paper electrophoresis (25 °C, Whatman 3 MM paper, 15 V cm⁻¹, 60 min, formic acid-acetic acid-H₂O (31:59:910), pH 2.0). [³H-Pro]-His-ProNH₂ (1.9 μmol; 1.21 μCi μmol⁻¹) was incubated in PBS (200 μl) at 37 °C. At the times, indicated aliquots (20 μl) of the reaction mixture were spotted on a sheet of Whatman 3 MM paper. After overnight chromatography in solvent 1, the areas corresponding to His-Pro (*R_f* = 0.73) were cut out and counted in a liquid scintillation counter.

Our work has demonstrated the presence in extracts of hamster hypothalamus⁴ and rat brain (unpublished observations) of the enzyme pyroglutamylpeptidase (L-pyroglutamyl peptide hydrolase, EC 3.4.11.8) which converts TRH to histidyl-prolineamide. This metabolite is unstable and readily cyclises to histidyl-proline diketopiperazine (His-Pro) (Fig. 1). The data in Fig. 2 document the instability of histidyl-prolineamide in phosphate buffered saline (PBS; 0.1 M potassium phosphate + 0.14 M NaCl, pH 7.2). ³H-proline-histidyl-prolineamide was prepared by incubation of ³H-proline-TRH with partially purified bacterial pyroglutamylpeptidase⁷. Incubation of ³H-proline-histidyl-prolineamide at 37 °C in PBS resulted in rapid cyclisation to the diketopiperazine. The half life of histidyl-prolineamide in these conditions was approximately 40 min. These data suggest that the pyroglutamylpeptidase-dependent conversion of TRH in brain to histidyl-prolineamide is probably followed by a rapid cyclisation to His-Pro.

The results of a further study (Fig. 3) provided additional evidence that His-Pro is an *in vivo* metabolite of TRH. [³H-Proline]-TRH (20 μl) was injected into the right lateral ventricle of a rat⁸. The animal was decapitated 5 min after the injection; the brain was then removed and extracted with 10 ml of 0.4 N HClO₄. Paper chromatography of the neutralised HClO₄ extract (see legend Fig. 3) revealed that, in addition to residual TRH, radioactivity was localised in acid TRH, linear histidyl-proline and His-Pro (approximately 8% of the total radioactivity). Incubation of acid-inactivated brain with radioactive TRH, followed by the same extraction procedure, indicated that TRH was not degraded non-enzymatically. The linear histidyl-proline was probably either derived from acid TRH by the action of pyroglutamylpeptidase or from histidyl-prolineamide by the

action of TRH amidase⁴. The scheme shown in Fig. 1 summarises our present theory on the pathway for TRH metabolism in brain. We have established the existence of enzymes forming acid TRH and histidyl-prolineamide⁴. Although the existence of an enzyme catalysing the conversion of histidyl-prolineamide to His-Pro seems likely, the instability of histidyl-prolineamide has, thus far, hindered out attempts to detect such an activity.

Breese *et al.*⁹ reported that TRH antagonises the effect of ethanol in inducing sleep in mice and rats; acid TRH did not substitute for TRH in this reduction of ethanol narcosis. Our findings that His-Pro is another metabolic of TRH prompted us to test its effect on ethanol narcosis. Figure 4 shows concentration curves for effectiveness of TRH and His-Pro in reducing ethanol-induced sleep. The data confirm the finding⁹ that TRH antagonises ethanol narcosis and that acid TRH is inactive. There was no effect on the time for loss of the righting reflex, but TRH significantly decreased the righting time. The more important finding, however, is that His-Pro is substantially more effective than TRH in this test. The data suggest that a reduction of sleep time to 50% of the control requires intraventricular injection of 1 μmol kg⁻¹ of His-Pro or 50 μmol kg⁻¹ of TRH. The study shown in Fig. 3 indicated that the intraventricular injection of 1 μmol kg⁻¹ of TRH leads to the formation of approximately 0.08 μmol kg⁻¹ of His-Pro. Figure 4 shows that 1 μmol kg⁻¹ of TRH or 0.08 μmol kg⁻¹ of His-Pro produce comparable reductions in ethanol-induced sleep. This observation is compatible with the notion that the activity of TRH in antagonising ethanol narcosis requires its conversion to His-Pro.

We previously introduced the hypothesis that the metabolism of TRH may generate compounds with biological

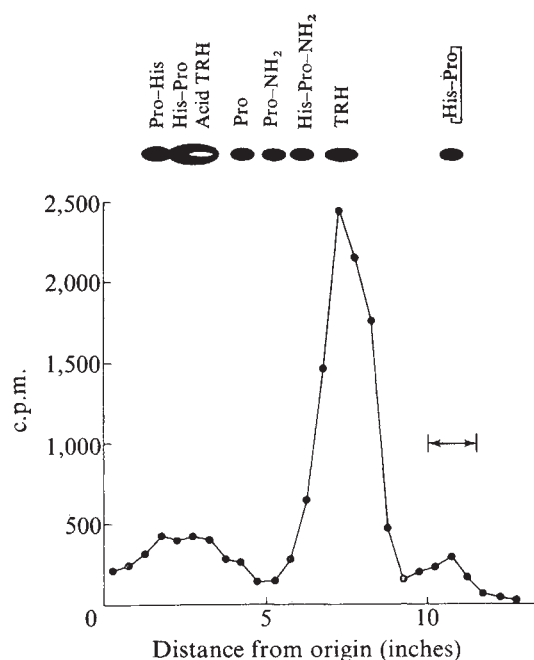


Fig. 3 Formation of His-Pro from intraventricularly administered TRH. A male Sprague-Dawley rat (approximately 100 g) was injected with 20 μl [³H-Pro]-TRH (10 μCi, 100 μCi μmol⁻¹) in saline. The animal was decapitated 5 min after injection, and the brain quickly removed. The brain was homogenised in 0.4 N HClO₄ (10 ml) and the centrifuged extract adjusted to pH 6.4–6.6 with saturated KHCO₃ solution. The supernatant solution was lyophilised and then extracted with a small volume of 90% methanol. The methanol extract was applied in a 30-cm band to a sheet of Whatman 3 MM paper and chromatographed (ascending) in solvent 1. The chromatogram was dried and a 1-inch strip was cut into 0.5 inch segments and counted. The area corresponding to His-Pro (10–11.5 inches from the origin) was extracted with methanol and rechromatographed (ascending) in chloroform-methanol-NH₄OH (60:45:20). The radioactivity comigrated with authentic His-Pro (*R_f* = 0.73).

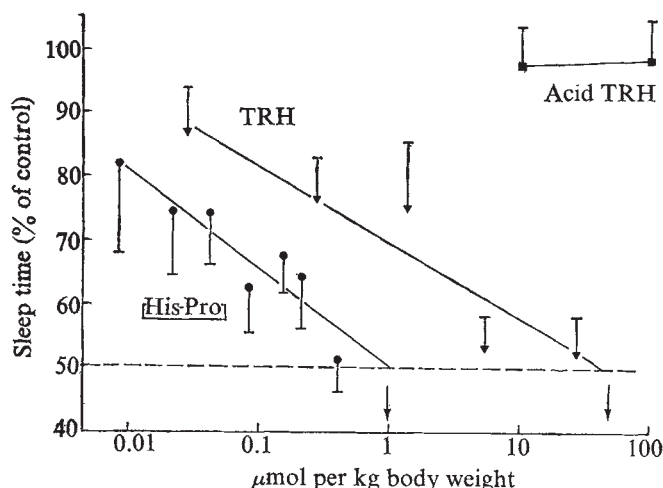


Fig. 4 Effect of TRH and His-Pro on sleep induced by ethanol. All animals were injected intraperitoneally with 8 ml ethanol (50% v/v in saline) per kg body weight. TRH, acid TRH or His-Pro (dissolved in saline) were administered to rats intravenicularly (15–18 μ l per animal) under light ether anaesthesia 10 min before the ethanol injection. The concentrations of the peptide solutions were such that 100 μ l contained the indicated number of μ mol. Sleeping time for animals was determined as described earlier⁶. The sleep time for control animals receiving saline intravenicularly and ethanol intraperitoneally was 69 ± 8 min ($n = 17$). Each point represents 6–10 animals (150–180 g) and the data are presented as mean $\pm$ s.e.m. The horizontal dashed line corresponds to a 50% decrease in the sleep time. The arrows point to the concentrations of His-Pro and TRH required to effect a 50% decrease in the sleep time induced by ethanol.

activity⁴ and the data presented here validate this idea. It will be of interest to see whether His-Pro corresponds to the 'active' form of TRH in a number of other systems where TRH has shown effects. It seems unlikely that TRH metabolism is obligatory for all of its effects since neither acid TRH nor His-Pro can replace TRH in reducing pentobarbital-induced sleep (preliminary experiments). Thus, the complete role of His-Pro in 'TRH-mediated' functions remains to be established.

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Eighty thousand β -adrenoreceptors in a single cell

CATECHOLAMINES function as hormones in peripheral organs and as neurotransmitters in the central nervous system (CNS). The primary event in the action of catecholamines is their interaction with specific α -adrenoreceptors or β -adrenoreceptors¹. The interaction of catecholamines with β -adrenoreceptors induces the activation of the enzyme adenylate cyclase which converts adenosine triphosphate (ATP) to cyclic AMP (ref. 2). Development of reliable radioligand binding methods has enabled the direct determination of the number of β -adrenoreceptors in various cell types^{3–12}. The content of β -adrenoreceptors in all the cells examined varies between a few hundred and a few thousand receptors per cell. These values correspond to $(0.2\text{--}2.0) \times 10^{-12}$ mol receptor per mg membrane protein, when membranes are prepared from these cells.

The absence of a rich source for β -adrenoreceptors had hindered attempts to identify and purify the receptor molecule. A report by Schubert *et al.*¹³ showed that skeletal muscle myoblasts (L6 cells) grown in culture respond to (–) noradrenaline by a 500-fold increase in their intracellular content of cyclic AMP and this finding prompted us to examine the β -adrenoreceptor density of these cells. We report here on the presence of 80,000 β -adrenoreceptors per cell in L6P cells grown in culture, as determined by a direct binding of ^{125}I (–)-iodohydroxybenzylpindolol (^{125}I (–)-HYP) to intact cells. In parallel, we examined the direct binding method by measuring ^{125}I (–)-HYP binding to intact turkey erythrocytes.

Rat skeletal myoblasts polyploid cells¹⁴ (L6P) were obtained from Dr D. Yaffe. The cells grown on collagen were detached from their support by incubation with ethylene diamine tetraacetate (EDTA) 1.5 mM for 2 h, washed and suspended in the isotonic buffer and ^{125}I (–)-HYP binding was measured. The experimental details are given in Fig. 1. The binding of ^{125}I (–)-HYP to intact turkey erythrocytes was also measured (Fig. 2) using similar conditions. Both L6P cells and turkey erythrocytes demonstrate a single class of ^{125}I (–)-HYP binding sites (Figs 1c and 2c) and exhibit the same affinity towards the radiolabelled β -blocker (Figs 1 and 2 and Table 1).

L6P cells were found to possess 80,000 ^{125}I (–)-HYP

Table 1 Number of β -adrenoreceptors in intact L6P cells and intact turkey erythrocytes

Cell type	Number of ^{125}I (–)-HYP binding sites per cell	^{125}I (–)-HYP receptor dissociation constant (M)	Ref.
L6P	80,000	1.74×10^{-10}	This study
Turkey erythrocytes*	1,100	1.60×10^{-10}	This study
Turkey erythrocytes	400–500	1.9×10^{-11}	12

* Turkey erythrocyte membranes were found to have 0.9 pmol receptor per mg membrane protein, using ^3H (–)-propranolol binding^{3, 4}. On the basis of this finding it was calculated that an intact turkey erythrocyte has 550 ± 120 receptors per cell³. Using equilibrium dialysis with ^3H (–)-isoproterenol, the number of specific β -adrenoreceptors was found to be $1,200 \pm 300$ receptors per cell³.

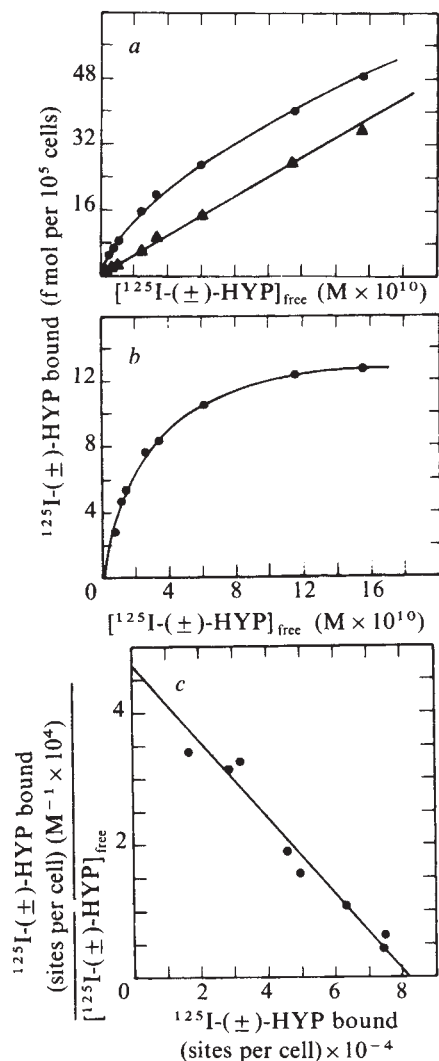


Fig. 1 Binding of $^{125}\text{I}-(\pm)\text{-HYP}$ to intact L6P cells. $^{125}\text{I}-(\pm)\text{-HYP}$ was prepared and purified as described previously⁹ and L6P skeletal muscle myoblasts were grown in plastic bottles for 9 d as described¹⁴. To detach the cells, the growth medium was removed and the clone incubated with 10 mM Tris-HCl pH 7.4 containing 1.5 mM Na_2EDTA and 140 mM NaCl at 37°C for 2 h. The surface was then scraped gently with a rubber stick to detach the cells from the surface. On exposure to the detaching buffer, the cells became round within 15 min as viewed under the microscope. The suspended cells were then centrifuged for 5 min at $950g$ and the washed cells were resuspended in 1.2 ml of the above buffer, counted and used for the $^{125}\text{I}-(\pm)\text{-HYP}$ binding assay. Fifty microlitres containing 1.0×10^5 cells ml^{-1} or 1.0×10^6 cells ml^{-1} were mixed with 0.45 ml of the buffer which contained increasing concentrations of $^{125}\text{I}-(\pm)\text{-HYP}$ ($0.2\text{--}15.6 \times 10^{-10}$ M) in the absence and in the presence of DL-propranolol (5.0×10^{-5} M final concentration). Thus the final concentration of cells in the binding mixture was 1.0×10^4 cells ml^{-1} in one set and 1.0×10^5 cells ml^{-1} in the second set. The binding mixtures were incubated for 30 min at 37°C . Subsequently 0.45 ml of each binding mixture was filtered on a Whatman GF/C glass filter as previously described^{9, 12} and counted in a gamma counter (Packard). A sample of 50 μl from each binding mixture was counted to determine the total $^{125}\text{I}-(\pm)\text{-HYP}$ concentration. From the values obtained for the bound $^{125}\text{I}-(\pm)\text{-HYP}$ and the total $^{125}\text{I}-(\pm)\text{-HYP}$ concentration in the binding mixture, the values of $[^{125}\text{I}-(\pm)\text{-HYP}]_{\text{free}}$ were computed. Each point represents the mean of two determinations. The data presented are for 1.0×10^5 cells ml^{-1} final concentration in the binding assay. *a*, Total $^{125}\text{I}-(\pm)\text{-HYP}$ bound; $\blacktriangle$, $^{125}\text{I}-(\pm)\text{-HYP}$ bound in the presence of 5.0×10^{-5} M DL-propranolol (nonspecific binding). *b*, Specific $^{125}\text{I}-(\pm)\text{-HYP}$ bound. *c*, Scatchard plot of data in *b*. The data in *b* were converted from fmoles per 10^5 cells to number of receptors per cell. Maximal binding was found to be 13.4 fmoles $^{125}\text{I}-(\pm)\text{-HYP}$ per 10^5 cells ($13.4 \times 6.03 \times 10^{23} \times 10^{-15}$ $^{125}\text{I}-(\pm)\text{-HYP}$ sites per 10^5 cells, which equals 80,400 receptors per cell; 6.03×10^{23} is Avogadro's number). Analysis of binding using 10^4 cells ml^{-1} yields 81,200 receptors per cell.

binding sites (Fig. 1), whereas turkey erythrocytes were found to possess 1,100 (Fig. 2). The latter value is close to the number of β -adrenoreceptors calculated on the basis of $^3\text{H}-(\pm)\text{-propranolol}$ binding to turkey erythrocyte membranes and to the value reported by Brown *et al.*¹², who measured $^{125}\text{I}-(\pm)\text{-HYP}$ binding to intact turkey erythrocytes (Table 1). The binding of $^{125}\text{I}-(\pm)\text{-HYP}$ in both cell types is inhibited by $(\pm)\text{-propranolol}$ (Figs 1 and 2), a specific β -adrenergic blocker, and is completed within 20 min of incubation. The maximal capacity of $^{125}\text{I}-(\pm)\text{-HYP}$ binding was found to be directly proportional to the cell concentration in the range of 10^4 cells ml^{-1} to 5×10^5 cells ml^{-1} for L6P cells, and in the range 1.0×10^6 to 5.0×10^7 cells ml^{-1} for turkey erythrocytes, confirming that equilibrium was reached between the cells and the radio-labelled ligand within the incubation time used.

In conclusion, it seems that the L6P cell line can become

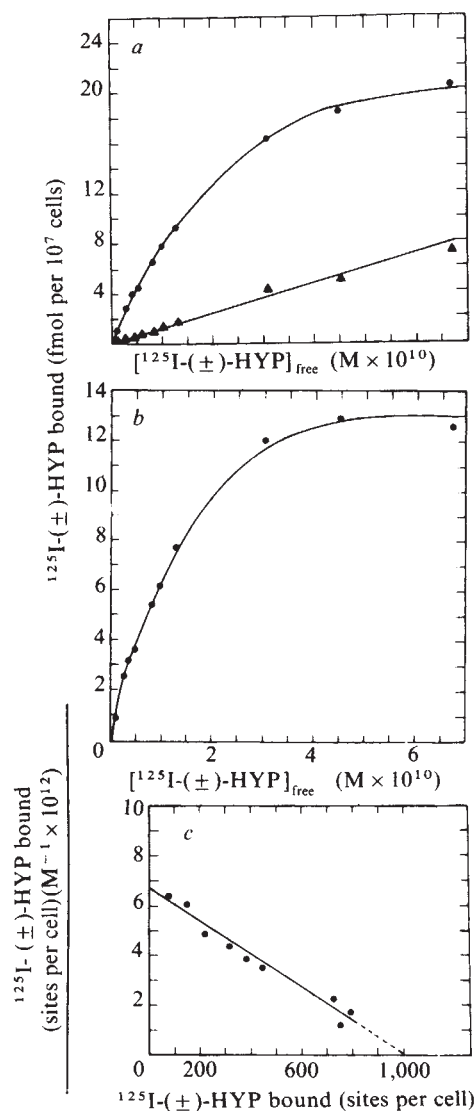


Fig. 2 Binding of $^{125}\text{I}-(\pm)\text{-HYP}$ to intact turkey erythrocytes. The preparation of a cell suspension of turkey erythrocytes essentially followed a published procedure^{12, 15}. Fresh heparinised blood from adult turkeys was centrifuged at $400g$ for 10 min at 4°C . This step was repeated four times. The washed erythrocytes were resuspended in 10 mM Tris-HCl pH 7.4 containing 0.14 M NaCl, 10 mM KCl, 11 mM glucose and 50 units ml^{-1} penicillin. A final concentration of 1.0×10^6 cells ml^{-1} and of 1.0×10^7 cells ml^{-1} were used for the binding experiments. The following steps were identical to those described in the legend to Figure 1. *a*, Total $^{125}\text{I}-(\pm)\text{-HYP}$ bound; $\blacktriangle$, $^{125}\text{I}-(\pm)\text{-HYP}$ bound in the presence of 5.0×10^{-5} M $(\pm)\text{-propranolol}$. *b*, Specific $^{125}\text{I}-(\pm)\text{-HYP}$ bound. *c*, Scatchard plot of data in *b*.

a good candidate for a rich source of β -adrenoreceptors. The access to such a source may advance the efforts aimed at the chemical characterisation of the β -adrenoreceptor and possibly its purification.

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How motoneurons control development of muscle tension

THE sum of all synaptic currents flowing at any moment through the motoneuron membrane represents the synthesis of the commands converging on to the final common path. Transduction of such commands into muscle contraction is accomplished by the conversion of current into motoneuronal firing and, in turn, conversion of the nervous discharge into mechanical events¹. Thus, the motor unit, that is, each motoneuron with the muscle fibres it innervates, operates as the interface between the central nervous system and the environment. The motor unit response to steady inputs is well known. Constant currents are transformed into constant-rate discharges, whose frequencies are proportional, over a definite range, to the current intensities²; in isometric conditions, such constant-rate discharges build up steady and proportional levels of muscle tension^{3–5}. On the other hand, little is known about the motor unit response to dynamic inputs. Recent work⁶, however, has suggested that the rate of growth of the current stimulating the motoneuron may be converted into a proportional rate of development of the muscle tension. In view of this last hypothesis, we have analysed the isometric tension developed by single motor units when motoneurons were stimulated with current pulses of trapezoidal shape, that is, reaching a given steady level after rectilinear (ramp) growth of different slopes. This approach was adopted on the assumption that current injected by an intracellular electrode into a motoneuron is physically equivalent of total synaptic current acting on the cell^{1,2}.

In cats the spinal cord was transected at high cervical level under sodium pentobarbital anaesthesia (40 mg per kg body weight), the lumbar cord was exposed and the tendon of the

gastrocnemius-soleus (G-S) muscle cut and attached to an isometric force transducer (Grass FT03). The muscle was stretched and maintained at the length giving the maximal isometric twitch response. The activity of G-S motoneurons was recorded intracellularly using glass microelectrodes (3M KCl) through which depolarising current pulses of trapezoidal shape (total duration 200–250 ms) were passed across the cell membrane. The rising phase of the pulses was rectilinear and the rise-time variable between 0 (rectangular pulse) and 120 ms. In some experiments, the trapezoidal currents were superimposed on a current step of rheobasic intensity. This experimental procedure has been successfully completed in nine motor units, all belonging to the fast type⁸ (time-to-peak of the single twitch: 18–25 ms). In eight of the nine the trials could be repeated for several different values of the steady-state current. Shape of the current pulses, motoneuronal discharge and isometric tension developed by the motor units were simultaneously displayed on the oscilloscope and photographed.

Constant-rate firing of a motoneuron can be obtained by two different methods, each leading to a different mode of muscle tension development. The motoneuronal discharge may be obtained by a train of individual short current pulses, each eliciting a single spike (Fig. 1*a*). Alternatively, rhythmic firing can be evoked by a long-lasting rectangular current step (Fig. 1*b*): in this case a constant-rate discharge of the same frequency as in Fig. 1*a* is reached through adaptation, that is, after the fall of the high instantaneous frequency of the first interspike interval. Such a frequency reduction is caused by summation of the conductance changes underlying the spike's afterhyperpolarisation (AHP) (refs 7–11). The related tension recordings show that muscular response is much faster and stronger in (*b*) than in (*a*). In (*b*) firing attains the same frequency as in (*a*) just after the second spike: the difference in tension seems therefore to be attributable to the initial, non-adapted, high-frequency discharge produced by the rectangular current. This suggests that modulation of the high-frequency, non-

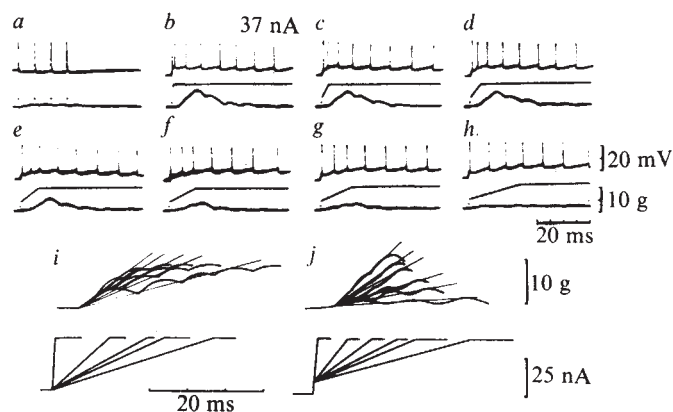


Fig. 1 Response of a motor unit to rectilinear input transients of different slopes. *b–h*, G-S motor unit of the fast type (time to peak for the single twitch 20 ms). For each recording, the middle trace represents the time-course of the motoneurone-stimulating current, the uppermost the neuronal discharge, the lowermost the mechanical response. Decrease in the slope of the current rising phase is accompanied by a parallel decrease in the peak initial tension and in the velocity of tension development. This is mediated by a rearrangement of the early phase of motoneurone firing, whose major feature is a progressive lengthening of the first interspike interval. With the slowest-rising ramp (*h*), firing frequency is nearly regular from the very beginning. Profile of isometric tension is, in this trial, similar to that obtained by single-pulse, constant-rate stimulation of motor cell (*a*). *i, j*, Upper traces are superimposed drawings of the tension profiles recorded in the same unit (*i*) and in another (*j*) fast G-S unit (single twitch time-to peak 25 ms) in which the amplitude of peak initial tension was not affected by the slope of stimulating current (lower traces). Straight lines connect the onset of tension with the points where 90% of peak tension is reached in each trial.

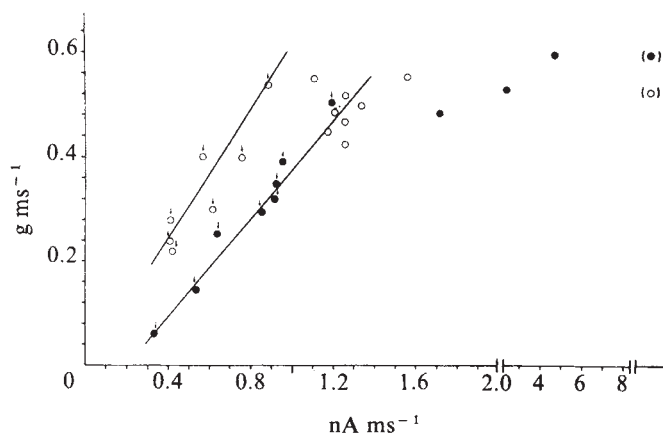


Fig. 2 Correlations between velocity of current commands to motoneurons and velocity of muscle tension recruitment. Same units as in Fig. 1j (○) and *i* (●). Abscissa: velocity of the rising phase of the currents injected into the motoneurons in nA ms^{-1} . Ordinate: velocity of development of isometric tension, calculated as the slope of the straight lines drawn in Fig. 1i and j. Points within brackets (on the right) signal the tension velocity developed by a rectangular pulse. Straight lines represent the regression functions calculated by the least squares method from the points marked by arrows. Note scale changes on abscissa.

adapted discharge may lead to a correlated modulation of the initial, transient phase of the tension profile.

The same motor unit was then stimulated with trapezoidal current pulses reaching the same level as in Fig. 1b, but after ramps of different slopes. Figure 1c–h shows that decreasing the ramp slope has a clear-cut effect on the initial part of the motoneuronal discharge but leaves steady-state firing untouched. The effect consists of a progressive increase in duration of the first interspike interval while the second and eventually (Fig. 1h) the third intervals undergo more complex changes. The relationships between changes in the ramp slope and rearrangement of the motoneuronal initial discharge will be analysed in detail elsewhere. It is of value here to follow the manner in which such slope changes modify the tension profiles. Figure 1c–h shows that a slowing down of the current rising phase is paralleled by a proportional reduction of both the absolute value of muscle tension and of its slope. This is more apparent in Fig. 1j, where the tension profiles from six records are superimposed.

Velocity of tension development was estimated from the experimental records by calculating the slope of the straight lines connecting the tension onset to the point at which 90% of maximal force was attained in each trial (Fig. 1j). This velocity has been plotted against the rise velocity of the related currents, as illustrated by the filled circles in Fig. 2. It is evident here that the two velocities are directly related to each other. Correlation seems to be linear over a considerable range of values, from about 0.3 nA ms^{-1} up to 1.2 nA ms^{-1} . Ramps steeper than 1.2 nA ms^{-1} do not increase the velocity of tension development much further (maximal velocity, attained with the rectangular current step, is represented by the dot within brackets). Such an insensitivity to the highest ramp slopes can be directly appreciated by comparing records (c) and (b) in Fig. 1.

In this motor unit, as well as in four other units in the present material (see also refs 5 and 12), tension responses to fast transients display an initial peak whose amplitude is positively correlated with ramp velocity (Fig. 1c–h). Such a peak might be important in non-isometric conditions, when an initial extra force may be required for quickly overcoming inertia at the start of the movement. In these units, the behaviour described above was less evident, or even disappeared, when the strength of the current pulse was increased: this produced a proportional increase of steady-state firing rate, which eventually became sufficiently high to keep the tension at (or close to) the level reached initially. Figure 1i refers to the response encountered in

the remaining four units and represents a series of tension profiles obtained from one of them and superimposed as in Fig. 1j. These profiles do not show any initial peak: whatever the strength of the current pulse, motoneuronal firing frequency at steady state (not illustrated) was always high enough to prevent the tension from falling after adaptation. In this unit, variations of the ramp slope are accompanied only by proportional changes in the slope of tension development. Velocity plot (Fig. 2, open circles) displays the same type of correlation described in the preceding example. This correlation was also consistently found in all other units investigated, independently of the presence or absence of the initial tension peak.

The linear transduction of the velocity of transients demonstrated here seems to depend on special properties of both components of the motor unit. Motoneurons encode velocity of the rising phase of a trapezoidal input signal by modulating the frequency of their initial, non-adapted discharge. The firing response to a current step represents the borderline example of such a modulation. It is accepted that the changes in firing frequency (that is, adaptation) observable in that condition are caused by the kinetics of summation of the AHP conductance changes^{10,13,14}. It therefore seems justifiable to assume that the same mechanism is also responsible for the modulation of motoneuronal discharge in response to ramps of different slopes.

At the muscular level, a feature which should prominently contribute to regulation of the rate of tension recruitment are the so called 'catch properties'¹⁵, that is, the strong 'potentiation' of the muscle twitch following the second of two short-interval stimuli^{15,16}.

It can be concluded that the motor unit is provided with a mechanism for independent transduction of both the steady levels and the transients of the input signals. While the steady level of isometric tension is proportional to the steady intensity of the current command to the motoneurone, velocity of force development can be regulated (over a definite range limited by the intrinsic muscle properties) by the command slope.

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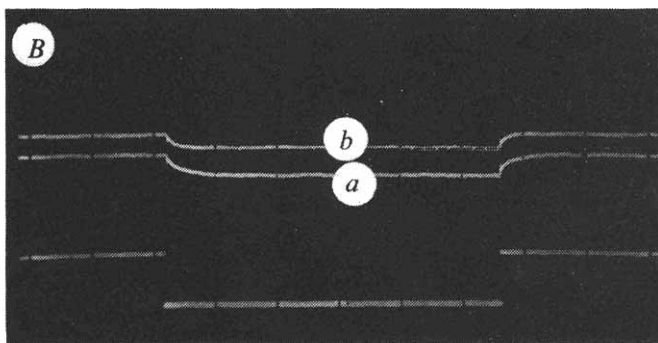
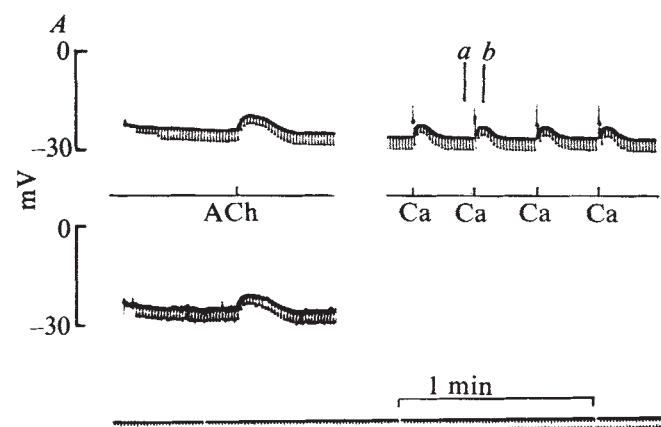
Acetylcholine-like effects of intracellular calcium application in pancreatic acinar cells

INDIRECT evidence suggests that Ca^{2+} is the mediator of the effects of acetylcholine and cholecystokinin-pancreozymin in pancreatic acinar cells. Sustained stimulation-evoked acinar fluid and enzyme secretion depends on extracellular Ca^{2+} (refs 1,2), stimulation causes uptake of $^{45}\text{Ca}^{2+}$ from the extracellular fluid³ as well as release of $^{45}\text{Ca}^{2+}$ from prelabelled glands with a dose-response curve very similar to stimulation-evoked membrane depolarisation and enzyme secretion⁴ and the Ca^{2+} ionophore A23187 induces enzyme secretion⁵ and

membrane depolarisation⁶. We have examined directly the bioelectrical effects of iontophoretic application of Ca^{2+} from intracellular microelectrodes in a mammalian gland cell preparation.

Two separate microelectrodes, one filled with either 0.2 M CaCl_2 (or sometimes 0.2 M MgCl_2) plus 0.05 M EGTA (ethylene glycol-bis- β (amino-ethylether)- N,N' -tetraacetic acid) (pH 7) or 3 M KCl and the other always filled with 3M KCl, were inserted very close to each other (20–50 μm intertip distance) into communicating surface acinar cells of mouse pancreas superfused *in vitro*. The tip of a third microelectrode, filled with 2M AChCl, was placed close to the impaled acinus in an extracellular position. The magnitude of iontophoretic currents was measured using a virtual ground current-voltage converter. Apart from one of the intracellular electrodes most often containing CaCl_2 , the set-up was identical to that used to determine the ACh null potential in pancreatic acinar cells⁷. The composition of the physiological saline solution flowing through the tissue bath was (mM): NaCl, 103; KCl, 4.7; CaCl_2 , 2.56; MgCl_2 , 1.13; NaHCO_3 , 25; NaH_2PO_4 , 1.15; D-glucose, 2.8; Na pyruvate, 4.9; Na glutamate, 4.9; Na fumarate, 2.7. The solution was gassed with 95% O_2 , 5% CO_2 .

Figure 1 shows the effects of extracellular ACh and intracellular Ca^{2+} applications in the same acinus. The effects of Ca^{2+} closely mimicked those of ACh causing membrane de-



membrane potential and resistance. A, The left-hand side of the trace shows the effect of a short pulse of iontophoretic ACh application (6×10^{-8} A, 0.5 s, retaining current 2×10^{-8} A) on membrane potential and resistance in two electrically coupled acinar cells. The upper trace represents the potential measured by an intracellular KCl electrode through which hyperpolarising rectangular current pulses (2×10^{-9} A, 100 ms duration) were injected repeatedly. The lower trace represents the membrane potential of a neighbouring cell recorded with a CaCl_2 electrode. At the point of interruption of the tracings, the CaCl_2 electrode was disconnected from the recording system and connected to a stimulator so that current could be injected through the electrode. At the marker signals labelled Ca, 5 nA, 0.5-s depolarising current pulses were applied through the Ca^{2+} electrode. B, The time course of membrane polarisation caused by injecting the 100-ms current pulse through the KCl electrode before and after the intracellular Ca^{2+} application (calibration: horizontal 20 ms, vertical 10 mV and 2×10^{-9} A).

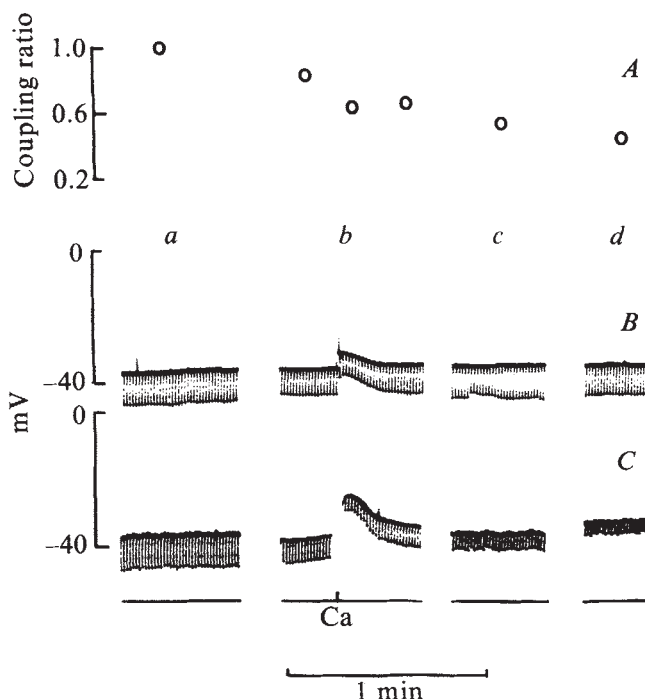


Fig. 2 The effect of repeated iontophoretic Ca^{2+} injections on acinar cell-to-cell coupling, membrane potential and resistance. *a-d*, Consecutive traces obtained from the same acinus with two indwelling electrodes in neighbouring communicating cells. *C*, Membrane potential measured by the CaCl_2 -filled microelectrode. Iontophoretic ejections of Ca^{2+} (20 nA, 500 ms duration) were repeatedly carried out (for example section *b*). Between *a* and *b* (2.5 min interval) three such Ca^{2+} ejections occurred, between *b* and *c* (1 min interval) two ejections took place and between *c* and *d* (1.5 min interval) one ejection was made. *B*, Membrane potential measured by the KCl microelectrode. Through this electrode, current pulses (hyperpolarising, 2.5 nA, 100 ms duration) were passed repetitively. *A*, Coupling ratios (magnitude of electrotonic potential change in cell impaled by CaCl_2 electrode, caused by the hyperpolarising pulses injected through the KCl electrode, divided by the magnitude of the current pulse induced potential change in the cell impaled by the KCl electrode) corresponding to the situations in *a-d*.

polarisation, reduction of input resistance and reduction of membrane time constant. Such ACh-like effects of intracellular Ca^{2+} applications (2–20 nA, 500 ms depolarising pulses) were repeatedly obtained in 14 cells from eight glands. Hyperpolarising current pulses of this magnitude had no effect.

Control experiments were carried out to exclude the possibility that these effects were unrelated to the intracellular Ca^{2+} application. The effects were not caused by the current passage or the potential change, since 2–20 nA depolarising or hyperpolarising current pulses applied through a KCl electrode never had any effect (six glands). Considerably larger current pulses (50–100 nA), however, caused membrane depolarisation and resistance reduction. These effects were observed after both depolarising and hyperpolarising current pulses, but the hyperpolarising pulses were by far the more effective. The shape of these potential changes was very different from those caused by Ca^{2+} injections. The depolarising phase was much steeper and the depolarisations were more short-lived, except in the case of the largest currents (> 100 nA). This phenomenon is probably caused by a reversible dielectric breakdown in the cell membrane (refs 8 and 9 and refs therein). The effects of Ca^{2+} injections were not evoked by releasing ACh from postsynaptic nerve endings surrounding the acini, since Ca^{2+} ejections with the CaCl_2 electrode extracellularly had no effect, probably reflecting the paucity of 'close' innervation¹⁰ and since the effects of intracellular Ca^{2+} applications were also observed during exposure of the gland cells to 1.4×10^{-6} M atropine (three glands) a concentration abolishing the effects of extracellular

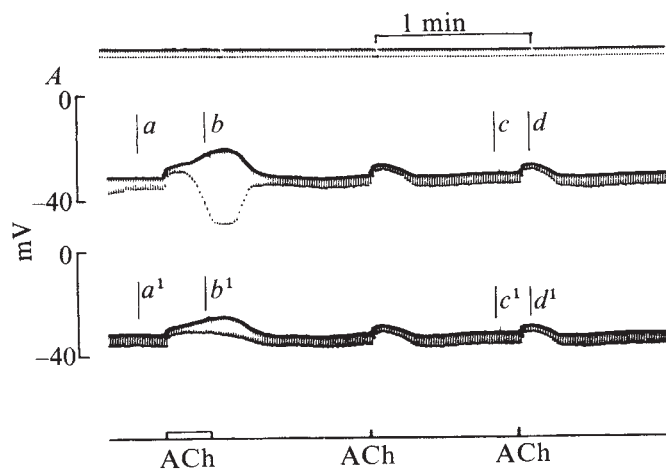
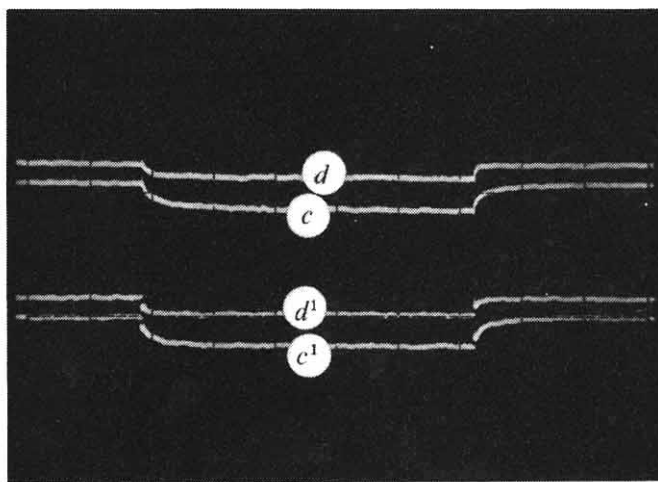
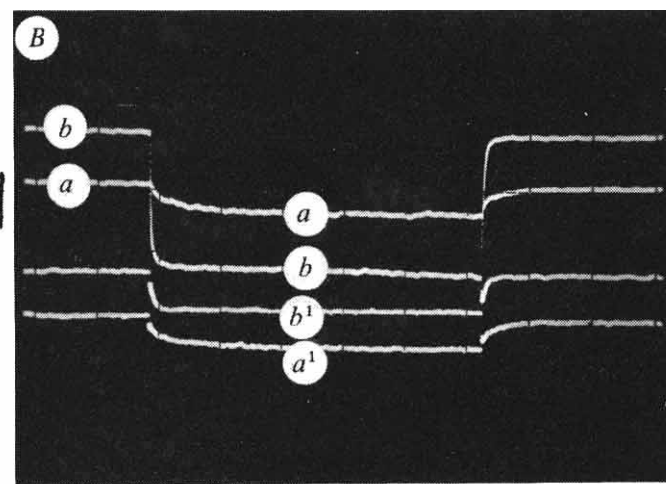


Fig. 3 The action of short and long pulses of ACh on membrane potential, resistance and electrical coupling. *A*, Two tracings of membrane potentials from two neighbouring acinar cells, both recorded with KCl electrodes. Hyperpolarising rectangular current pulses (2×10^{-9} A, 100 ms) were repetitively injected through the electrode recording the potential represented by the upper trace. The event marker signals represent the duration of the ejecting ACh pulses from the third extracellular microelectrode (6×10^{-8} A, retaining current 2×10^{-8} A). *B*, The shape and time course of the membrane polarisations in both cells caused by the current injections (calibration: horizontal 20 ms, vertical 10 mV).



iontophoretic ACh stimulation. The results of Ca^{2+} injections were not unspecific divalent cation effects since 2–20 nA, 500 ms depolarising current pulses applied through MgCl_2 -filled electrodes (four glands) never caused any changes. Dielectric breakdown was observed after large current pulses (50–100 nA, both depolarising and hyperpolarising).

It is well known that intracellular Ca^{2+} injections can cause uncoupling of epithelial cells which are normally fully electrically coupled¹¹. Small short-lived Ca^{2+} pulses resulting in the potential and resistance changes seen in Fig. 1 did not cause uncoupling. It was not possible to use much larger pulses since these evoked dielectric breakdown. After 5–10 min of repetitive Ca^{2+} injections in six glands, however, when it was still possible to get reliable recordings from the CaCl_2 electrode, uncoupling (that is, a reduction in the magnitude of electrotonic potential changes picked up by the CaCl_2 electrode caused by current injection through the KCl electrode in the neighbouring cell) was observed (Fig. 2). Uncoupling was never observed after Mg^{2+} or K^+ ejections.

The demonstration that intracellular Ca^{2+} applications result in membrane depolarisations similar to those evoked by ACh suggests, but does not prove, that the ACh effects are caused by a rise in cytosol Ca^{2+} concentration. Unfortunately the small size of pancreatic acinar cells (10–15 μm in diameter) has prevented the use of photoproteins (such as aequorin) to monitor changes in intracellular Ca^{2+} concentration following cell activation^{8,11,12}. It does seem, however, that uncoupling is always caused by a rise in ionised Ca^{2+} concentration¹¹. It is therefore of interest that prolonged extracellular ACh application can evoke reversible acinar cell uncoupling (Fig 3). It can also be seen in Fig. 3 that at the stage of ACh-evoked uncoupling, the depolarisation in one cell (the cell into which current pulses are injected) becomes more marked than in the neighbouring cell. When the cells subsequently recouple the

effects of short doses of ACh become equal. Such ACh-evoked acinar cell uncouplings were observed repeatedly in four different glands. This phenomenon, which had been postulated on the basis of indirect evidence¹³, is probably the most direct demonstration of an ACh-evoked increase in pancreatic acinar cytosol Ca^{2+} concentration available at present.

The new experimental findings that ACh can cause acinar cell uncoupling and that intracellular Ca^{2+} applications can mimic the effect of ACh provide new evidence for the hypothesis that ACh acts primarily on membrane Ca^{2+} translocation¹⁴.

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Nuclear localisation of an oocyte component required for the stability of injected DNA

Most cells contain deoxyribonucleases but do not degrade their nuclear DNA. This paradox is sometimes explained by assuming that the nucleases do not have access to DNA, from which they may be separated by the nuclear membrane. Another possibility is that cells contain a component which stabilises DNA even in the presence of nucleases. We show here that such a component is present in the frog oocyte, a type of cell in which deoxyribonucleases have been repeatedly demonstrated^{1,2}. Although we have not characterised this component, we show that it is localised in the nucleus; it seems to be absent from cytoplasm but may leak into it when the nuclear membrane is ruptured. The DNA whose stability we have investigated is a small circular molecule introduced into the oocyte by microinjection, but the conclusions probably apply also to the endogenous nuclear DNA of the oocyte, and perhaps to the nuclear DNA of eukaryotic cells in general.

We performed three types of experiment in which *Xenopus laevis* oocytes were injected with purified ³H-labelled SV40 DNA and then analysed singly by methods which show whether the injected DNA lay within the nucleus or in cytoplasm. First, using autoradiography, we defined the location of ³H-SV40 DNA immediately after injections aimed at the oocyte nucleus (germinal vesicle, GV). Second, individual nuclei and cytoplasm, manually separated, were analysed for their content of ³H-SV40 DNA several hours after injections aimed at either the GV or vegetal pole cytoplasm. Third, the molecular configuration of this recovered DNA was correlated with its location in each oocyte.

Autoradiographs were prepared from sections of 20 oocytes fixed within 5 min of injections aimed at the GV. A common result was that much of the injected DNA was located in an area to one side of the GV (Fig. 1a, b). In a few cases the labelled DNA was situated exclusively in the cytoplasm. Most significant for this discussion, however, was the fact that in about half of the analysed oocytes, some of the labelled DNA had been deposited clearly inside the GV and had already started to mix with its contents (Fig. 1c, d).

In the second type of experiment, oocytes whose nuclei contained ³H-SV40 DNA were identified by a method allowing subsequent analysis of the molecular configuration of the injected DNA. Injections of ³H-SV40 DNA were directed either at the GV or into vegetal pole cytoplasm. The GVs were manually removed 24 or 48 h later and ³H-DNA from each GV and cytoplasm was extracted and counted as indicated in the legend to Fig. 2. The results, shown in Fig. 2, demonstrate three points. First, from each oocyte the total radioactivity recovered in acid insoluble form was variable, but the mean for all injected oocytes was almost the same as the total injected radioactivity. Similar recoveries were obtained from oocytes after injection both into the nuclear region (Fig. 2a) and into vegetal pole cytoplasm (not shown). Second, ³H-DNA injected into the cytoplasm did not penetrate the GV, even after 48 h (Fig. 2c). Third, oocytes injected so as to aim for the GV fell into two classes (Fig. 2b). In about half, more than 95% of the recovered DNA was present exclusively in the cytoplasm, a distribution similar to that in oocytes intentionally injected into the cytoplasm. Clearly in these oocytes the injection had either missed the GV or completely leaked out of it, and we refer to them as 'GV-miss' oocytes. In the remainder, referred to as 'GV-hit' oocytes, substantial quantities of ³H-SV40 DNA were present in the

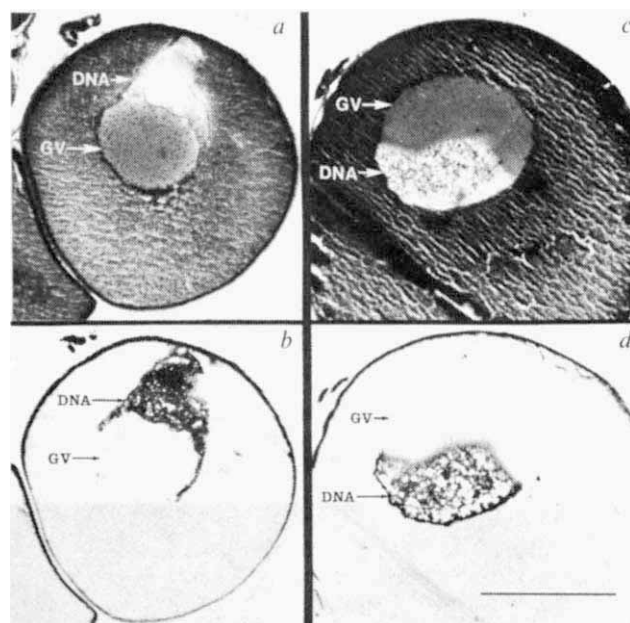


Fig. 1 Intracellular distribution of ³H-SV40 DNA immediately after injection into oocytes. ³H-SV40 DNA (50 nl containing 5 ng) was injected into oocytes¹², aiming for the GV. They were fixed 2–5 min later, for sectioning and staining (a, c) and subsequent autoradiography (b, d). In the oocyte shown in a and b, the injected DNA is located outside the GV. In the oocyte shown in c and d, the injected DNA is clearly located within the GV, and is already beginning to mix with its contents. Scale bar, 400 μ m.

GV, in most cases exceeding 80% of the total recovered DNA.

Finally we investigated the configuration of injected DNA recovered from isolated GVs and cytoplasm by methods described in the legend to Fig. 3. Each GV-injected oocyte was classified as 'GV-hit' or 'GV-miss' by counting aliquots of the extracts of nucleus and cytoplasm as described above, and the configuration of the DNA in each oocyte was then correlated with its intracellular distribution. At the time of injection most of the ³H-SV40 DNA was covalently closed and supercoiled (form I), with a small proportion in nicked circular form (form II) (Fig. 3a).

DNA deposited directly in the cytoplasm of oocytes was always degraded. It appeared in gels as faint but discrete bands corresponding to the full length linear (form III) and nicked circular (form II) configuration, together with smears representing partially digested linear molecules (Fig. 3e); form I DNA was never observed. The absence of any residual covalently closed DNA was confirmed by alkali sucrose gradient analysis of pooled aliquots from these cytoplasmic samples. In most 'GV-miss' oocytes, the DNA recovered from cytoplasm showed a gel distribution closely similar to that of oocytes intentionally injected into the cytoplasm: it was extensively degraded (Fig. 3d).

'GV-hit' oocytes showed a strikingly different pattern. First, all the intranuclear ³H-SV40 DNA appeared in gels as a single, sharp form I band; no linear DNA was ever observed (Fig. 3b). Second, detectable quantities of ³H-SV40 DNA were sometimes found in the cytoplasm of these oocytes and it is of special interest that in such cytoplasmic samples almost all the ³H-DNA was also in a supercoiled (form I) configuration (Fig. 3c). Nicked and linear forms of ³H-DNA were absent or present only in traces. Alkali sucrose gradient analysis of pooled aliquots of 'GV-hit' cytoplasmic samples confirmed the presence of conserved covalently closed ³H-SV40 DNA.

These results show that DNA deposited inside the GV was conserved in supercoiled form, whereas DNA injected

directly into cytoplasm was degraded. We interpret the unexpected conservation of DNA in the cytoplasm of 'GV-hit' oocytes as due to leakage of nuclear contents into the cytoplasm. There is direct evidence that such leakage occurred: visibly ruptured GVs were recovered from six GV-injected oocytes, all of which showed form I DNA in their cytoplasm. It seems probable that GV rupture was caused by the large volume of DNA solution injected (50 nl, that is, approximately 1.4 times the volume of the full grown oocyte GV).

Two conclusions may be drawn from these experiments. First, conservation of injected DNA is due, not to its separation from cytoplasmic nucleases by some impermeable membrane, but to a component which renders it stable even in the presence of nuclease. This is demonstrated by the conservation of DNA within the cytoplasm of oocytes in which the GV had been ruptured by injection (cytoplasm analysis of 'GV-hit' oocytes): this conclusion concurs with the observed presence of endonucleases within eukaryotic nuclei³. Second, the oocyte component responsible for DNA stability is normally localised in the GV.

At this stage, we can make only general observations about the nature of this GV component. It could be a nuclease inhibitor, or a type of molecule which combines with DNA so as to render it resistant to nucleases. We have found that, when conserved, the injected DNA is

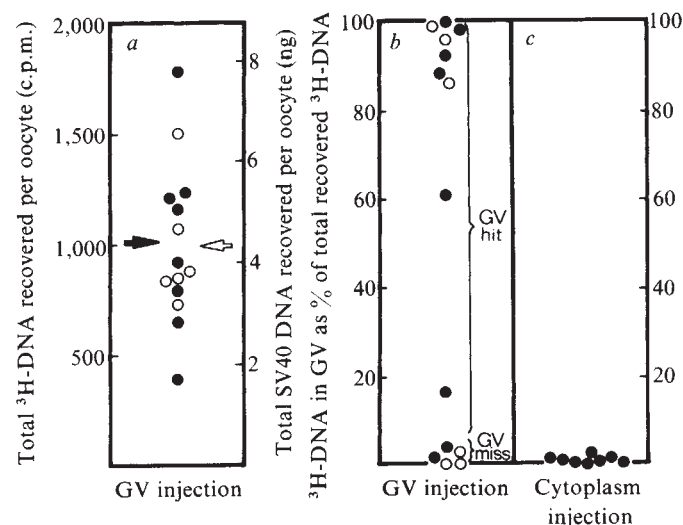


Fig. 2 Location of ³H-SV40 DNA recovered from single oocytes 24 h (○) or 48 h (●) after injection. (a) shows, for each oocyte, the total recovered ³H-DNA counts (and hence the total mass of recovered SV40 DNA), taking GV and cytoplasm together. The left hand arrow indicates the mass and radioactivity of DNA in each injection, estimated by delivering the usual oocyte injection volume through the microinjection pipette directly on to a glass chip which was then counted in scintillant. The right-hand arrow shows the mean recovered mass of DNA from these oocytes. (b) Refers to oocytes injected aiming for the GV, and (c) to oocytes injected in vegetal pole cytoplasm; in both b and c the counts in each GV are expressed as a percentage of the total counts recovered from the oocyte. Oocytes were injected with approximately 50 nl medium containing 5 ng purified ³H-SV40 DNA, specific activity 380,000 d.p.m. μg⁻¹. After incubation at 19 °C in MBS-H medium¹² for 24 or 48 h, follicle cells were manually peeled off and the GVs were manually removed and washed free of yolk. Each GV and cytoplasm was frozen within 30 s of isolation. ³H-DNA was extracted by making a homogenate of each GV or cytoplasm in 100 μl buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 0.5% sodium dodecyl sulphate, 0.75 mg ml⁻¹ proteinase K and 0.8 mg ml⁻¹ yeast tRNA (as carrier). The homogenate was incubated at 37 °C for 2 h and extracted twice in chloroform : isoamylalcohol (24 : 1). The aqueous phase was precipitated in 10% trichloroacetic acid at 0 °C on to Millipore filters which were dried and counted with an efficiency of about 60% in scintillant.

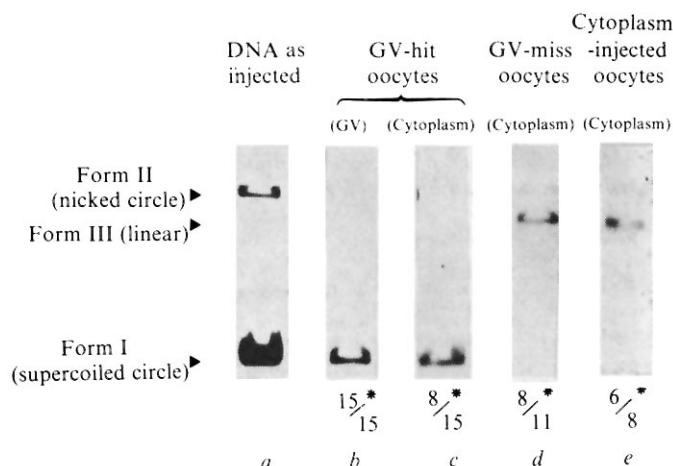


Fig. 3 Conformation of DNA injected into oocytes and recovered from isolated GVs and cytoplasm. GV-injected oocytes, classed as 'GV-hit' or 'GV-miss' as described in Fig. 2, are compared with oocytes injected in vegetal pole cytoplasm. The gel tracks show typical examples of the conformation adopted by ³H-SV40 DNA in the GV of 'GV-hit' oocytes and in the cytoplasm of oocytes of all classes. GVs of 'GV-miss' and cytoplasm-injected oocytes contained negligible amounts of ³H DNA and were not analysed on gels. Seven of fifteen cytoplasm from 'GV-hit' oocytes and two of eight cytoplasm from cytoplasm-injected oocytes differed from the pattern shown in that they contained little ³H DNA and showed no discrete gel bands. Three of eleven cytoplasm of 'GV-miss' oocytes also differed from the pattern shown in that they contained form I DNA. At the time of isolation the GV of one of these oocytes was found to be merely a ruptured membranous sac and we presume that, in the other two cases also, GV material had leaked into cytoplasm. Individual GVs or cytoplasm were homogenised and extracted in chloroform: isoamylalcohol as described in the legend to Fig. 2. An aliquot was removed for scintillation counting to permit GV-injected oocytes to be classified as 'GV-hit' or 'GV-miss', and the remainder was loaded on 1% Agarose slab gels for electrophoresis¹³ at 40 mA for 14 h. Thereafter the gel was impregnated with PPO, dried and fluorographed¹⁴. *Proportion of samples with the gel pattern shown

transcribed^{4,5} and is assembled into a nucleoprotein complex whose buoyant density and regularly repeated substructure are those expected of chromatin (A.H.W., J.B.G. and R. A. Laskey, in preparation). However, assembly into chromatin might be a consequence, rather than cause, of DNA stability. In addition to histones^{6,7} several other proteins are strongly localised in the GV of oocytes^{8,9}, and might stabilise DNA.

The behaviour of this GV component at nuclear division is of interest. It is probably released into the cytoplasm when the oocyte nuclear membrane breaks down at the time of maturation into eggs; uncleaved eggs, unlike oocytes, conserve DNA injected into their cytoplasm¹⁰. This type of component may, we suggest, be of general occurrence in eukaryotic cells, protecting chromosomal DNA from nuclease attack throughout the cell cycle and especially during mitosis.

Conceivably, cytoplasmic nucleases could be of value to cells, ensuring the breakdown of any exogenous DNA taken into the cell. Uptake of exogenous DNA occurs rather frequently as a result of cell death: in the course of the normal cell renewal in many tissues, the products of dead cells—including their DNA—are ingested by their viable neighbours¹¹. Phagocytes also may require protection from ingested bacterial DNA. Viral DNA however may be resistant to degradation by virtue of its own core proteins.

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Rigid control of synthesis of collagen Types I and III by cells in culture

THE constituents of the extracellular matrix have a fundamental role in the structure and function of all tissues. A major insight into the composition of this matrix came with the discovery^{1–3} that interstitial collagen was not homogeneous, but rather composed of two species, termed Type I and Type III. Type I collagen (composed of two $\alpha 1(I)$ chains and one $\alpha 2$ chain) usually forms densely packed fibrils (the cross-banded collagen commonly seen in electron micrographs). In comparison, Type III collagen (composed of three $\alpha 1(III)$ chains) exists, in part, as reticulin, in which fine, randomly dispersed collagen fibrils are intimately associated with other connective tissue components^{3,4}. Appreciation of the possible functional significance of the heterogeneity of the interstitial collagens has come from the analysis of the ratio of Type I to Type III collagen in different tissues in health and disease. Most pliable tissues (skin, intestine, blood vessels, lung) have a I:III ratio of 2–3 to 1, while rigid tissues (bone, tendon) contain only Type I collagen^{5,6}. Studies of inflammation and wound healing have demonstrated that early granulation tissue (a material with little tensile strength) has a very low I:III ratio^{4,7}, while mature scar (a stiff tissue) has a high I:III ratio⁸. In addition, disorders associated with a loss of tissue compliance (arteriosclerosis⁹, pulmonary fibrosis¹⁰) are associated with a shift in the normal I:III ratio toward relatively more Type I collagen. Taken together, these findings suggest that the structure and function of tissues are intimately associated with the relative amounts of the interstitial collagens, with Type I tightly packed and tough and Type III loosely associated and pliable. If the relative amounts of collagens I and III are as important as this data suggests, the maintenance of normal interstitial tissues must be critically dependent on cells in the local environment rigidly maintaining their differentiated state with respect to the synthesis and degradation of these collagen types. We describe here a study designed to evaluate this hypothesis, using a model system to assess the 'tightness' of cellular control of the synthesis of collagens I and III by a population of similar cells (fibroblasts in culture) over approximately 20 replicative cycles.

To accurately assess the relative rates of synthesis of Types I and III by cells in culture, we adapted cyanogen bromide (CNBr) mapping techniques to quantitate all of the collagen in the culture, regardless of its type, form or location. To increase the accuracy of the quantitation of both collagen types, we used NB-6, a diploid fibroblast derived from the newborn rabbit lung, since it synthesises

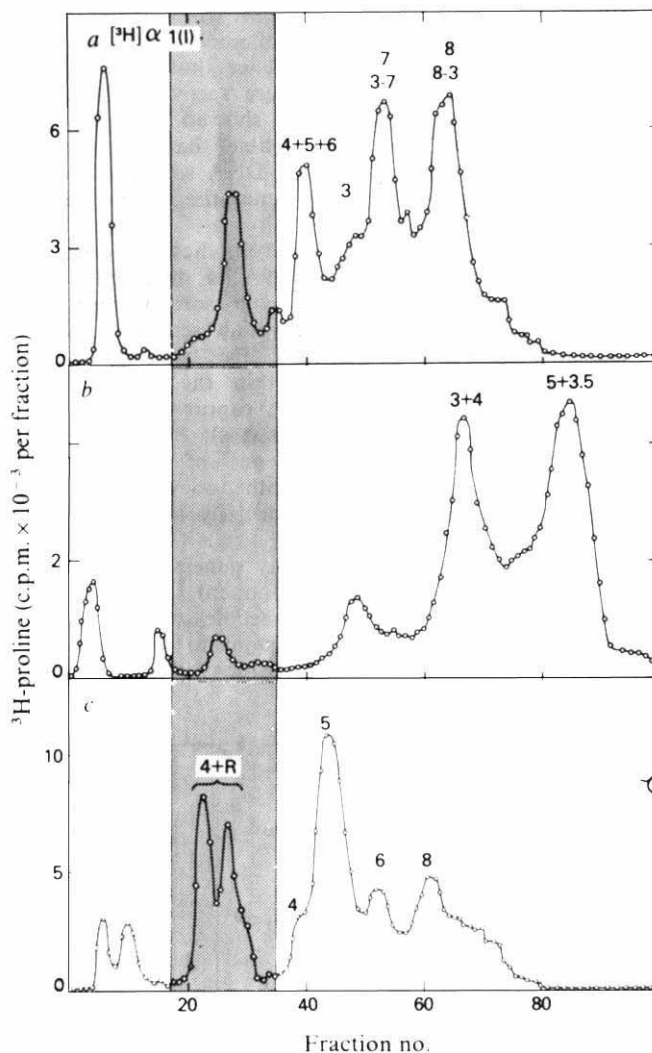


Fig. 1 Quantitation of the ratio of Type I to Type III collagen synthesised by rabbit lung fibroblasts (NB-6) in culture, step 1: carboxymethyl-cellulose (CMC) chromatography of CNBr peptides. NB-6 cells were grown to confluency in 100 mm² plates, rinsed with phosphate-buffered saline (PBS), and labelled for 24 h at 37 °C in Dulbecco's modified Eagle's medium (DMEM) 10 ml per plate, containing 0.3 mM ascorbic acid, 0.5 mM β -aminopropionitrile and either 50 μ Ci ¹⁴C-proline (260 mCi mmol⁻¹) per plate or 1.5 mCi ³H-proline (32 Ci mmol⁻¹) per plate. Isolation, morphological characterisation and subcultivation of the NB-6 culture was as previously described¹¹. Medium was collected, dialysed against 10 mM Tris-HCl, pH 7.4, 2 M urea, and ³H-proline labelled Type I and Type III collagens were prepared using diethylaminoethylcellulose chromatography^{11,16}. The ³H-proline labelled Type I collagen was further purified into ³H- $\alpha 1(I)$ and ³H- $\alpha 2$ chains by CMC chromatography¹⁷. Using these methods, the labelled Type III collagen, Type I collagen and $\alpha 1(I)$ and $\alpha 2$ chains are greater than 98% pure¹¹. To approximate the conditions in which the collagens in an entire tissue culture plate would be quantitated, labelled collagens were added to pooled cells and medium from five unlabelled culture plates, dialysed against 0.1 M acetic acid, lyophilised and CNBr peptides were produced using an excess (100 mg) of CNBr. The CNBr peptides of the purified collagens or their component chains were separated by CMC chromatography (0.9 $\times$ 12 cm column; 250 ml $\times$ 250 ml, 10 mM to 160 mM NaCl gradient in 20 mM Na-citrate, pH 3.8, flow rate 40 ml h⁻¹; 5 ml fractions) and aliquots (0.2 ml) of each fraction were counted in Aquasol. *a*, CNBr peptides of ³H- $\alpha 1(I)$ chains synthesised by NB-6 cells in culture; the characteristic peptides are labelled. *b*, CNBr peptides of ³H- $\alpha 2$ chains from the same cultures; $\alpha 2$ CNBr (3.5) is probably peptide 3–5; it has a molecular weight^{17,18} of approximately 60,000. *c*, CNBr peptides of ³H-Type III collagen. The shaded area ('region A') was used to quantitate the ratio of Type I to Type III collagen synthesised by NB-6.

significant quantities of both collagens I and III (ref. 11).

NB-6 cultures were labelled with ^3H -proline in the presence of ascorbic acid and β -aminopropionitrile at sub-cultivations 5 and 10 at different stages in the cell growth curve. The cultures were labelled for 18 h (in the conditions used, NB-6 cells synthesise collagen in a linear fashion for >24 h) and the resulting ^3H -proline labelled proteins solubilised with CNBr. To quantitate the ratio of ^3H -Type I to ^3H -Type III collagen synthesised by the cultured cells, peptides characteristic of ^3H -Type I and ^3H -Type III collagens were then isolated by carboxymethyl-cellulose chromatography followed by isoelectric focusing gels (Figs 1 and 2). Region A (shaded area Fig. 1) was chosen for the I:III quantitation for two reasons: (1) it is the least complex region of the Type I and Type III CNBr peptide map, containing only one peptide from Type I [$\alpha 1(\text{I})\text{CB3}$] and two peptides from Type III [$\alpha 1(\text{III})\text{CB4} + \alpha 1(\text{III})\text{CB(R)}$] (Fig. 2a); and (2) the I and III peptides in region A are clearly separated from each other by isoelectric focusing (Fig. 2b); this procedure is quantitative and easily reproducible. The final ratio of ^3H -Type I to ^3H -Type III synthesised by the cells in culture was determined from a standard curve prepared from known mixtures of ^3H -Type I and ^3H -Type III previously purified from NB-6 cultures (Fig. 2c).

This method obviates a number of technical problems

inherent in the quantitation of collagen types synthesised by cells in culture: (1) the use of a standard curve circumvents possible differential losses of I and III during analysis and takes into account the differences in proline+hydroxyproline residues in the I and III peptides used for quantitation; (2) CNBr cleavage of the entire plate ensures that all of the newly synthesised I and III will be quantitated, independent of whether the collagens are in solution in the culture medium, precipitated on the cell layer, or intracellular and also independent of the extent of processing from procollagen to collagen; (3) to ensure that ^3H -Type I and ^3H -Type III are hydroxylated to the same extent and not contaminated by non-collagen proteins, the isoelectric focusing gels are evaluated by quantitating both ^3H -proline and ^3H -hydroxyproline in the I and III peptides; and (4) since cross-linked peptides may map differently, β -aminopropionitrile is added to the cultures in concentrations which inhibit cross linking of the newly synthesised collagens¹¹.

The NB-6 cells synthesise collagen Types I and III in a ratio of 1.94 ± 0.09 (mean $\pm$ s.e.m.) to 1 (that is, 66% I, 34% III) over at least 20 replicative cycles (Fig. 3). Thus in conditions in which the extracellular milieu is defined and constant, the fibroblast exhibits remarkably tight control over the relative synthesis of these two collagen types. This holds true even through periods of rapid cell

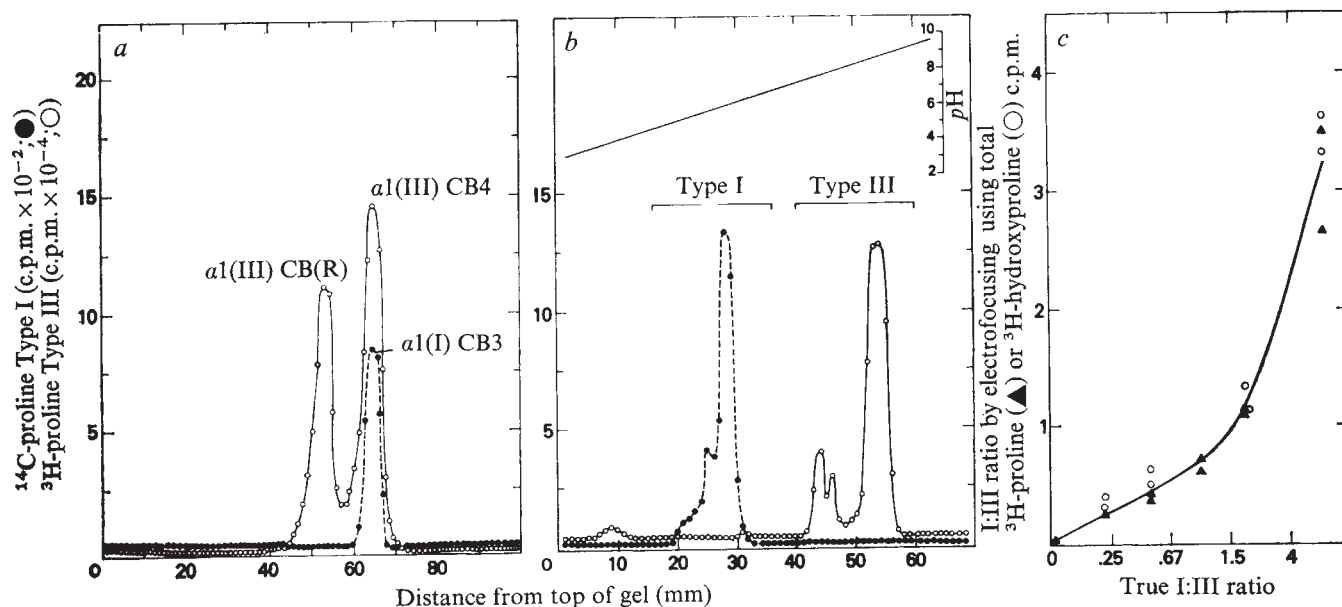


Fig. 2 Quantitation of the ratio of Type I to Type III collagen synthesised by NB-6 cells in culture, step 2: analysis of ^3H -labelled CNBr peptides from region A of the CMC chromatogram. A mixture of ^{14}C -Type I and ^3H -Type III CNBr peptides were chromatographed on CMC. The A region peptides were precipitated with ethanol (-20° , 18 h, using 500 μg of rabbit skin Type I collagen CNBr peptides as carrier), isolated by centrifugation (72,000g, 2 h, 4°C). a, ^{14}C -Type I (●) and ^3H -Type III (○) peptides from region A isolated on sodium dodecyl sulphate (SDS)-acrylamide gels. The peptides were dissolved in 1% SDS, 1% β -mercaptoethanol in 0.1 M phosphate buffer, pH 7.4 and electrophoresed as previously described¹¹. Region A contains one Type I peptide $\alpha 1(\text{I})\text{CB3}$ of approximately 12,000–13,000 molecular weight and two Type III peptides [$\alpha 1(\text{III})\text{CB4}$ and $\alpha 1(\text{III})\text{CB(R)}$]; $\alpha 1(\text{III})\text{CB4}$ electrophoreses with $\alpha 1(\text{I})$ and $\alpha 1(\text{III})$ peptides, there are no significant $\alpha 2$ peptides found in region A. b, ^{14}C -Type I (●) and ^3H -Type III (○) peptides from region A separated by isoelectric focusing gels. Five-tenths ml of a concentrated acrylamide solution [made up of 3 ml of 30% acrylamide–1% bis-acrylamide; 0.3 ml pH 3–10 Ampholytes (LKB); 0.7 ml ammonium persulphate (15 mg ml $^{-1}$)] was added to 1 ml of the A region CNBr peptides at 4°C , the solution vortexed and used to form a 67 $\times$ 5-mm cylindrical gel. The gels were focused at 1 mA per gel (0.2 M phosphoric acid anode solution, 0.2 M NaOH cathode solution) until 200 V was reached, then 200 V for 5 h total²⁰. The pH of the focused gels was measured using a micro pH electrode (Gel Pro-pHler, Bio Rad) and confirmed by determining the focusing point of standard proteins²¹. After focusing, the gels were fractionated at 1 mm intervals (Gilson Gel fractionator) and fractions were counted in Aqualul. Alternatively, gels were sectioned at appropriate intervals, hydrolysed in 6 M HCl and the labelled hydroxyproline quantitated¹¹. The ^{14}C -Type I peptide [$\alpha 1(\text{I})\text{CB3}$] focused at between pH 4.5 and 6.5; and ^3H -Type III peptides [$\alpha 1(\text{III})\text{CB4} + \alpha 1(\text{III})\text{CB(R)}$] focused between pH 7.1 and 8.3. c, Various mixtures of ^3H -Type I collagen and ^3H -Type III collagen synthesised by NB-6 cultures were treated with CNBr along with a fixed amount of ^{14}C -Type III collagen (used to locate region A on CMC) and unlabelled cells and medium from five culture plates. Each mixture was chromatographed on CMC, region A pooled (as determined from the elution pattern of ^{14}C -Type III; see Fig. 1c) ethanol precipitated and electrofocusing. The Type I region (pH 4.5–6.5) and Type III region (pH 7.1–8.3) was fractionated and the ^3H -proline and ^3H -hydroxyproline d.p.m. quantitated. Abscissa: the known ratio of ^3H -Type I and ^3H -Type III mixed together before CNBr cleavage. Ordinate: the I:III ratio quantitated with isoelectric focusing of CMC region A: ▲, total ^3H c.p.m. in the Type I region/total ^3H c.p.m. in the Type III region; ○, ^3H -hydroxyproline in the Type I region/ ^3H -hydroxyproline in the Type III region. Similar results are obtained using either total ^3H c.p.m. or ^3H -hydroxyproline. In an actual experiment quantitating the ratio I:III synthesised by NB-6 in culture, the c.p.m. of ^3H -hydroxyproline in the Type I region is compared to the c.p.m. of ^3H -hydroxyproline in the Type III region and the true I:III ratio read off the abscissa of the standard curve.

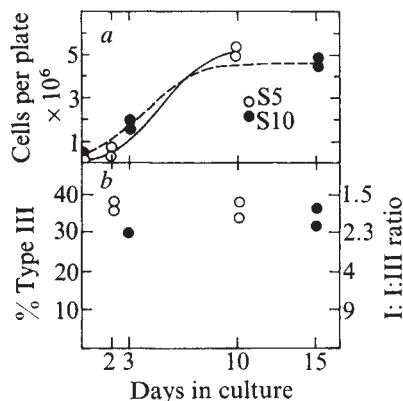


Fig. 3 Quantitation of the ratio of Type I to Type III collagen synthesised by NB-6 cells at two different subcultivations (S5 and S10) and at different times within each subcultivation (early log through late confluency). NB-6 cells in the 4th and 9th subcultivation were detached, counted and plated at a density of 2×10^5 cells per 100-mm² plate in 25 ml DMEM containing 10% foetal calf serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 5 µg ml⁻¹ fungizone. The medium was replaced every 4th day. For quantitation of the ratio of Type I to Type III collagen synthesised, the NB-6 cells were incubated for 18 h at 37 °C in 4 ml DMEM containing 0.3 mM ascorbic acid, 0.5 mM β-aminopropionitrile and 500 µCi ³H-proline (32 Ci mmol⁻¹). After incubation, the cells were detached by scraping the plates, the cells and medium from five plates were combined, dialysed against 0.1 M acetic acid and lyophilised. Thereafter, the methods described in the legends to Figs 1 and 2 were used. An average of 90% of the ³H-hydroxyproline in the plates was solubilised with CNBr. As a check on the number of cells replicating in the plates at each time, separate plates were incubated under identical conditions with 10 µCi per plate of ³H-methylthymidine (65 Ci mmol⁻¹) for 1 h at 37 °C. After incubation, the cells were washed with PBS, detached, counted and the incorporated ³H-thymidine quantitated. For subcultivation 5, cells at day 10 incorporated 0.1% ³H-thymidine per cell of that incorporated by cells at day 2; for subcultivation 10, cells at day 15 incorporated 0.4% ³H-thymidine per cell as did cells at day 3. *a*, Cell number at early log (days 2–3), late log-early confluency (day 10) and late confluency (day 15) are shown for cells in subcultivation 5 (○) and 10 (●). *b*, The ratio of Type I-to-Type III collagen synthesised by NB-6 cells at the same subcultivations and days in each subcultivation as in (*a*). Within the limits of the method, the ratio of I:III was invariant at the times studied with an average of % Type III of 34.1 ± 1.0 (I:III 1.94 ± 0.09).

synthesise the major interstitial components in a carefully controlled fashion.

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Long term culture of tumour-specific cytotoxic T cells

MANY investigators have been successful in the maintenance of long term tissue culture of human bone marrow-derived (B) cells. These cell lines have been established from both normal subjects¹ and from patients with lymphoproliferative disorders². In most cases, long term B-cell lines have been shown to harbour the Epstein-Barr virus genome which some investigators feel is required for establishment and maintenance of long-term cultures³. There are fewer reports describing continuous culture of human thymus derived (T) cell lines, and when successful, the lines have only been established from patients with acute lymphocytic leukaemia⁴. Although these cell lines have been shown to bear surface markers of normal human T lymphocytes, there have been no reports which suggest that they possess the ability to respond to immunologic stimuli or to differentiate into antigen-specific lymphocytes. Cytotoxic murine T cells have been kept in continuous culture only through repetitive mixed-lymphocyte stimulation⁵. In contrast to long term human lymphocyte lines, these cells proliferated only when stimulated with allogeneic lymphocytes and eventually died after a few weeks in culture. Morgan, Ruscetti and Gallo recently reported a method by which medium conditioned by phytohaemagglutinin-stimulated normal human lymphocytes allowed for the selective long-term growth of normal T cells⁶. In contrast to the previously mentioned cell lines, the proliferation of these reported T-cell cultures was totally dependent on the presence of an exogenously-produced growth factor supplied by the conditioned medium. In this report we describe an adaptation of this method which allows the long term culture of antigen-selected cytotoxic T cells which continue to demonstrate high levels of syngeneic tumour-specific cytotoxicity after more than 4 months in culture.

Cytotoxic T cells were generated in secondary allogeneic mixed tumour-lymphocyte cultures (MTLC). Spleen cells were harvested from normal C57B1/6 mice and from C57B1/6 mice immunised with allogeneic (H-2^d) F4-5 Friend virus (FLV)-induced leukaemia cells (from Dr W. Ostertag, Max Planck Institute, Göttingen, Germany). Both spleen cell populations were co-cultured with mitomycin-treated (100 µg ml⁻¹ for 60 min)⁷ F4-5 cells in Click's medium (Altick Associates) with 2% heat inactivated foetal calf serum (FCS) for 5 d (res-

division. For example, on day 2 of passage 5 the incorporation of ³H-thymidine per cell was 1,000-fold greater than at day 10 of passage 5; even so, the I : III ratio of newly synthesised collagens was identical at both days (Fig. 3). Since 20 population doublings should be ample time for different clonal subpopulations to become apparent¹², these results also suggest, at least for NB-6, all cells in the culture are synthesising collagens I and III at approximately the same ratio. If different clones are characterised by different I : III ratios, the relative number of these clones remains constant in conditions of a defined extracellular milieu.

These results do not imply that fibroblasts never alter the relative amounts of collagen types that they synthesise. In special circumstances when cells become senescent^{13,14}, or when the extracellular milieu changes, such as in inflammation¹⁵, these cells could change the relative proportions of collagens I and III that they produce, either by altering the differentiated characteristics of each cell or by selecting specific clones emphasising one collagen type. The data presented, however, indicate that in conditions of a constant extracellular environment, the relative ratio of the two known interstitial collagens synthesised by a population of fibroblasts is invariant. Thus, the cells most concerned with the maintenance of interstitial structures are programmed to preserve the status quo by continuing to

ponder: stimulator ratio of 40:1). Viable cells collected from primary allogeneic MTLC were restimulated with mitomycin-treated F4-5 cells and placed back in culture for a further 2 d. Cultures of cytotoxic cells generated in secondary allogeneic MTLC of normal C57B1/6 spleen cells were designated cytotoxic lymphoid line 1 [CTLL(1)] and cells collected from repetitive allogeneic MTLC of spleen cells from C57B1/6 mice immunised with F4-5 were designated CTLL(2). The population of cytotoxic lymphocytes collected from secondary allogeneic MTLC was a heterogeneous one in that one lymphoid subpopulation was reactive against allogeneic targets while a second subpopulation contained lymphocytes capable of effecting syngeneic tumour-specific cytotoxicity¹³.

Viable cells collected from secondary allogeneic MTLC were placed in continuous culture in 50% Click's medium and 50% growth factor. Growth factor was prepared from 48-h tissue culture medium (RPMI 1640 (GIBCO) with 10% FCS) from cultures of concanavalin A (con A, 2.5 $\mu\text{g ml}^{-1}$, Miles-Yeda) stimulated normal DBA/2 spleen cells. Growth factor was filtered through a 0.2- μm filter (Nalge-Sybron) to remove subcellular particles before use in long-term culture medium.

Both CTLL(1) and CTLL(2) have remained in continuous proliferative culture for 22 weeks. Representative growth curves for both lines are shown in Fig. 1. Cells seeded at a concentration of 5×10^3 per ml reach a saturation density of approximately 3×10^5 cells per ml after 6 or 7 d in culture thereby demonstrating a doubling time of 24 h. Proliferative growth is totally dependent on the presence of growth factor. CTLL(1) and CTLL(2) cannot be maintained in continuous culture in either Click's medium supplemented with FCS or in RPMI 1640 medium supplemented with FCS and con A.

Cells from cultures of CTLL(1) and CTLL(2) were used periodically as effector cells in lymphocyte-mediated cytotoxicity (LMC) assays⁸, against the allogeneic tumour target cell, F4-5 (Fig. 2) and the syngeneic FLV-induced FBL-3 (Hn), (Fig. 3). Both F4-5 and FBL-3 (Hn) were confirmed as non-virus-producing murine leukaemia cells by reverse transcriptase⁹ and X-C assays¹⁰. When LMC data is expressed in terms of lytic units per 10^6 effector cells ($\text{LU} \times 10^{-6}$), the allogeneic cytotoxicity mediated by CTLL(1) increased from $76.9 \text{ LU} \times 10^{-6}$ after 3 weeks in culture to $555 \text{ LU} \times 10^{-6}$ after 17 weeks in culture. Similarly, allogeneic lysis effected by CTLL(2) rose

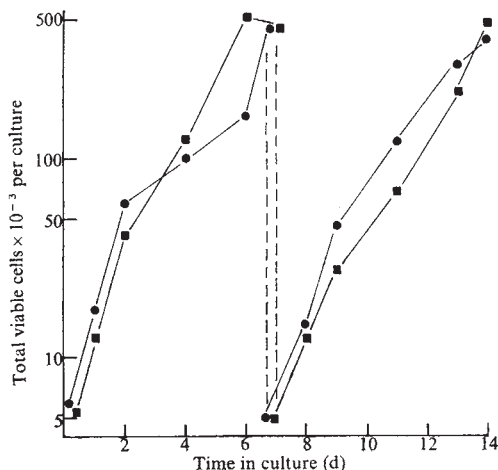


Fig. 1 Representative growth curves of CTLL(1) (●) and CTLL(2) (■). Dotted lines indicate passage of long term cell lines and reseeding of additional culture flasks at the day 0 cell concentration of 5×10^3 cells per ml. Both cell lines exhibited this pattern of proliferative growth provided they were grown in the presence of medium containing 50% growth factor. Click's medium with 50% growth factor was also supplemented with 2% heat-inactivated FCS, 300 $\mu\text{g ml}^{-1}$ fresh L-glutamine, 50 units ml^{-1} penicillin, 50 $\mu\text{g ml}^{-1}$ streptomycin, 16 $\mu\text{mol ml}^{-1}$ NaHCO_3 and 25 $\mu\text{mol ml}^{-1}$ HEPES buffer. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C.

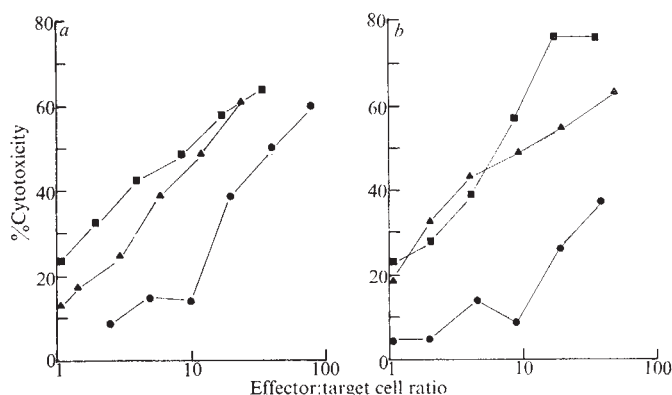


Fig. 2 *a*, Allogeneic cytotoxicity demonstrated by CTLL(1) against the FLV-induced non-producer murine leukaemia cell, F4-5, after 3 weeks (●), after 10 weeks (▲), and after 17 weeks (■), in continuous culture. *b*, Cytotoxicity demonstrated by CTLL(2) against F4-5 after 3 weeks (●), 10 (▲) and 17 weeks (■) in culture. Per cent cytotoxicity was determined in lymphocyte-mediated cytotoxicity assays which measured Cr^{51} release of radiolabelled target cells. Target cells were labelled with 250 μCi of $\text{Na}_2\text{Cr}^{51}\text{O}_4$ (specific activity 100–400 mCi per mg Cr, Amersham-Searle). Target cell concentrations were adjusted in RPMI 1640 with 10% FCS to 0.1 c.p.m. per cell. Target cells (100 μl) were mixed with log₂ dilutions of effector cells collected from CTLL(1) and CTLL(2) long term cultures, in V-bottom microplates (1S MVC-96-TC, Linbro Scientific). The Cr-release reaction was stopped after a 4-h incubation at 37 °C by a 10-min 300g centrifugation at 4 °C. c.p.m. of Cr-release were determined by counting 100 μl of each well supernatant on a liquid scintillation counter, per cent specific lysis was determined using the following equation

%Specific lysis=

$$\frac{\text{experimental c.p.m.} - \text{medium control c.p.m.}}{\text{maximum release c.p.m.} - \text{medium control c.p.m.}} \times 100$$

Medium control c.p.m. were obtained from 100- μl supernatant aliquots sampled from microplate wells containing 100 μl of target cells and 100 μl of RPMI 1640 with 10% FCS. Maximum release c.p.m. were generated from microplate wells containing 100 μl of target cells and 100 μl of Zap Isoton Solution (three drops added to 5 ml double distilled H_2O), Coulter Electronics. Lytic units were defined as the number of effector cells necessary to cause 30% specific lysis. Growth factor was not present in LMC assays.

from $40 \text{ LU} \times 10^{-6}$ after 3 weeks of culture to $500 \text{ LU} \times 10^{-6}$ 14 weeks later. Results of LMC assays (Fig. 3) indicate that the syngeneic tumour cell lysis mediated by CTLL(1) and CTLL(2) was tumour specific. Although FBL-3 (Hn) murine leukaemia cells were effectively lysed, syngeneic normal cells (C57B1/6 lymph node and con A-stimulated (2.5 $\mu\text{g ml}^{-1}$) spleen cells remained unaffected). Syngeneic tumour-specific cytotoxicity demonstrated by CTLL(1) effector cells increased from $10 \text{ LU} \times 10^{-6}$ after 3 weeks in culture to $333 \text{ LU} \times 10^{-6}$ after 17 weeks in culture. CTLL(2)-mediated cytotoxicity of FBL-3 (Hn) cells rose from $16.7 \text{ LU} \times 10^{-6}$ following 3 weeks in culture to $142.9 \text{ LU} \times 10^{-6}$ after 17 weeks of continuous *in vitro* passage.

Figure 4 shows Wright-Giemsa-stained preparations of CTLL(1) and CTLL(2) after 22 weeks of continuous proliferation *in vitro*. Both cell lines are blastoid and highly vacuolar in appearance, and were lysed by anti-theta serum (AKR anti-C3H thymus from Litton Bionetic) when tested using absorbed rabbit complement¹¹. Histochemical stains of both CTLL(1) and CTLL(2) for peroxidase, periodic acid Schiff, naphthal chloroacetate and α -naphthylacetate esterases, were negative. These tests confirm that the syngeneic tumour-specific cytotoxicity demonstrated by CTLL(1) and CTLL(2) was mediated by cytotoxic T cells maintained *in vitro* with the aid of a growth factor produced by con A-stimulated spleen cells.

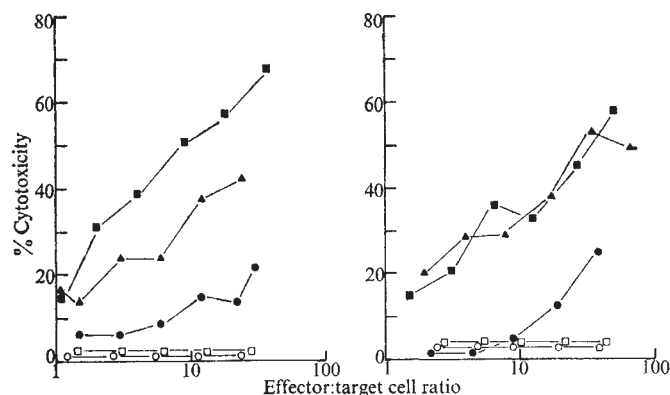
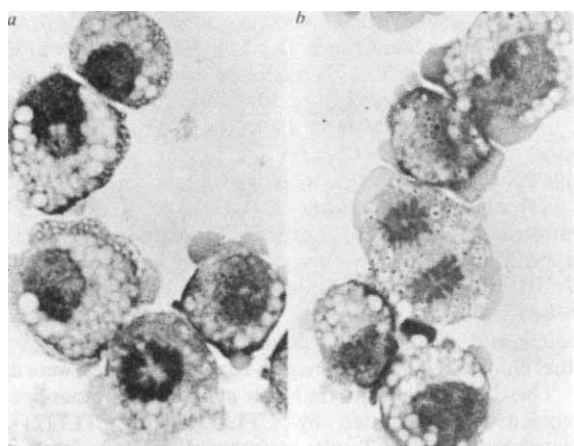


Fig. 3 *a*, Cytotoxicity of syngeneic FBL-3 (Hn) Friend leukaemia cells effected by CTLL(1) cells after 3 (●), 10 (▲) and 17 weeks (■) in culture. Cytotoxicity demonstrated by CTLL(1) against syngeneic normal lymph node (○) and con A-stimulated spleen cells (□). *b*, Cytotoxicity of FBL-3 (Hn) by CTLL(2) cells after 3 (●), 10 (▲), and 17 weeks (■) in culture. CTLL(2)-mediated cytotoxicity of syngeneic normal lymph node (○) and con A-stimulated spleen cells (□). FBL-3 (Hn) was adapted to continuous *in vitro* culture from ascites-passaged FBL-3 cells, obtained from Dr Ronald Herberman, NCI. LMC assay conditions, and radiolabelling of all target cells identical to those described in Fig. 2.

The continuous proliferation of differentiated T lymphocytes *in vitro* raises questions as to the regulatory factors operating *in vivo* which prevent continuous clonal expansion after antigenic stimulation. The demonstration that continuous proliferation of cytotoxic T cells *in vitro* is possible, suggests that some humoral or cellular factor may be operating *in vivo* to prevent differentiated proliferation. Furthermore, the long term culture system described here might serve as a starting point for the identification and characterisation of these growth promoting and inhibiting, regulatory factors.

Finally, although cytotoxic lymphocytes generated *in vitro* have been shown to be effective mediators of tumour cell lysis *in vivo*¹², the inability to generate these cells in large numbers has prevented their widespread use. The ability to propagate large populations of syngeneic tumor antigen-specific cytotoxic lymphocytes through the use of a growth factor, makes possible the testing of these lymphocytes in adoptive immunotherapy. The syngeneic cytotoxic specificities of the long-term T-cell lines tested in this study were directed against non-producer leukaemia cells. Because murine non-producer leukaemia cells represent the model system most analogous to human leukaemia, this study provides evidence that the *in vitro* generation of large numbers of leukaemia-specific human cytotoxic T cells might be possible.

Fig. 4 Wright-Giemsa-stained cytocentrifuge preparations of *a*, CTLL(1) and *b*, CTLL(2) cells after 22 weeks of continuous culture *in vitro*. ($\times 6,000$).



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Effect of glucose and insulin on collagen secretion by human skin fibroblasts *in vitro*

INCREASED glucose concentration in the medium in which normal fibroblasts are grown in culture caused an increase in one type of procollagen synthesised, an increase in the incorporation of labelled carbon from glucose into collagen, and an increase in the rate of collagen secretion. The addition of insulin to the culture media did not affect collagen secretion.

Skin fibroblasts growing *in vitro* synthesise collagen when the medium is supplemented with ascorbic acid¹. Most of this collagenous protein remains soluble in the medium as procollagen types I and III (refs 2-5). The procollagens are secreted by the fibroblasts into the medium where they are eventually cleaved, polymerised and deposited as extracellular insoluble collagen fibrils. Human fibroblasts *in vitro* do not efficiently convert procollagen into collagen, so there is an accumulation of intact procollagen in the medium⁶.

Skin biopsy material was obtained from human subjects undergoing surgery for non-metabolic disorders. Fibroblasts were isolated after trypsinisation, and grown as monolayers in minimum essential medium (MEM, Flow Labs) plus 10% foetal calf serum. Ascorbic acid ($50 \mu\text{g ml}^{-1}$) was added to the culture medium to ensure maximal collagen synthesis⁷. Cells were continuously passaged and monitored periodically for their ability to incorporate tritiated thymidine into DNA (ref. 8). All the experiments reported here were performed on cells at confluency. For 24 h before the experiment, the cells were placed in a serum-free medium (MEM) plus 1% non-essential amino acids. After 24 h, fresh MEM + 1% non-essential amino acids were added and glucose and/or insulin (0.1 U ml^{-1}) was added to the experimental dishes. The medium was removed 24 h later and analysed for collagen-like material according to the technique of Paz and Gallop⁹. Briefly, collagen was isolated by TCA precipitation, hot TCA solubilisation and collagenase (purified by chromatography)¹⁰ treatment and determined by micro-biuret analysis using rat-tail collagen as standard. In some experiments, hydroxyproline was determined by the method of Bergman and Loxley¹².

To demonstrate that the material isolated in our assay system contained collagen or procollagen, samples were

collected after collagenase digestion, desalted by cation exchange column, and hydrolysed in 6 M HCl for 24 h at 100 °C. Amino acids were analysed on a Beckman 121 M amino acid analyser. In selected experiments, protein labelled with ^3H -proline was characterised by DEAE-52 cellulose chromatography¹³. Cells remaining in the dish after removal of the medium were washed, solubilised with alkali, and protein¹⁴ and DNA¹⁵ determinations carried out.

Cells growing in a high glucose medium secreted more collagen than those in glucose at a physiological concentration (Table 1). The addition of insulin did not increase the synthesis or secretion of collagen at glucose concentrations of 1 mg ml⁻¹ or 3 mg ml⁻¹ in the medium. Insulin did, however, stimulate the accumulation of non-collagen protein in the monolayer cells. We conclude that the increase in cell protein (data not given) is part of an insulin stimulation of growth. In the range of glucose concentrations of 1–5 mg ml⁻¹, increasing amounts of collagen were found in the media of the fibroblasts, but above this range no further increase was noted (Table 2).

DEAE-52 cellulose chromatography of the labelled medium collected after 24 h of high and normal glucose treatment gave a radioactive profile similar to that described by others^{2,3,9,16}. Normally we find three peaks; first a very small peak which is native collagen, a second large peak which is procollagen type I and a third peak which is presumably procollagen type III. In glucose-treated cultures there is a substantial (30–50%) increase in the third peak. Salt-extracted ^3H -labelled collagen from the cellular material remaining attached to the culture chamber gave identical DEAE-52 profiles with 24 h of 1 mg ml⁻¹ or 3 mg ml⁻¹ glucose treatment.

Table 1 Effect of glucose and insulin on the amount of collagen secreted by fibroblasts into the medium

Experiment	Control	Glucose (3 mg ml ⁻¹)	Insulin (0.1 U ml ⁻¹)	Glucose + insulin
1	0.066	0.115	0.047	0.100
2	0.131	0.193	0.141	0.197
3	0.152	0.207	0.185	0.227
4	0.102	0.139	0.053	0.147
5	0.086	0.182	0.068	0.167
Mean	0.107	0.167	0.097	0.168
± s.e.m.	0.015	0.017	0.027	0.022

The amount of collagen secreted into the serum-free medium by fibroblasts during a 24-h period expressed as μg collagen per μg monolayer cell protein in each culture dish. Collagen was measured in duplicate by microbiuret assay against rat-tail collagen. Total monolayer cell protein per dish was determined by dissolving the monolayer in alkali and using an aliquot of this in standard protein determination against bovine serum albumin. In these experiments the amount of protein in each culture dish remained fairly constant, the amount of collagen increased by 50% after the addition of glucose. Insulin had no effect. The data are the mean of five experiments ± the standard error of the mean.

Amino acid analysis of extracted collagen demonstrated twice as much hydroxyproline in each sample of the high glucose medium than in the normal. Colorimetric analysis for hydroxyproline gave similar results; for example 24 μg collagen in the control medium and 36 μg collagen in the glucose-treated medium.

We wondered whether the increased secretion was a result of increased cell proliferation, however, in our growth conditions neither the incorporation of tritiated thymidine in DNA nor the total DNA content was increased by raising the glucose concentration of the medium. In addition, there was no visible evidence (using phase-contrast microscopy) of increased proliferation of fibroblasts in high glucose media. There was no significant difference in cell protein or cell counts with increased glucose concentration in the medium. Rheinwald and

Table 2 Effects of glucose concentration on collagen secretion by fibroblasts

Experiment	Glucose concentration (mg ml ⁻¹)				
	1	2	3	5	7
1	0.049		0.081	0.131	0.149
2	0.058		0.080		
3	0.072	0.108	0.138		

The effect of glucose concentration on the amount of collagen secreted into the medium by fibroblasts, expressed as μg collagen in the medium per μg monolayer cell protein as described in Table 1. There is a dose-dependent response of human diploid fibroblasts to increased glucose concentration in the medium. At glucose concentration of 5 mg ml⁻¹ or higher, the stimulation of collagen accumulation in the medium began to level off. The amount of protein associated with the monolayer remained constant.

Green¹⁷ also reported no increase in cell density at high glucose levels.

In one experiment, the incorporation of ^{14}C from glucose into collagen was measured. The amount of label incorporated into collagen was 85% greater in cells grown in medium containing 3 mg ml⁻¹ glucose than in cells grown in 1 mg ml⁻¹. The addition of insulin did not increase the incorporation of label into collagen. We are now trying to determine whether the labelled carbon residues are in the polypeptide chain or in the disaccharide portion of collagen.

Rechler and Podskalny¹⁸ have demonstrated insulin receptors in fibroblasts but many investigators cannot demonstrate insulin effects in cultured cells. Shaw and Amos¹⁹ have shown that fibroblasts deprived of glucose become highly responsive to insulin; however, cells growing at ≥ 1 mg ml⁻¹ glucose do not respond to insulin by increased glucose uptake. At these glucose concentrations therefore, glucose transport into the cells is gradient dependent and insulin independent. In our experiments the addition of insulin did not cause an additive effect on collagen secretion.

These data suggest that the ambient glucose concentration of fibroblasts can influence the amount, the type, and possibly the glycosylation of collagen secreted by these cells. The implications of this are of great importance to the diabetic patient. Recently, a highly glycosylated haemoglobin molecule, HbA_{1c}, has been found in increased amounts in poorly controlled diabetics²⁰. Basement membranes isolated from kidney glomeruli of diabetic patients show a marked increase in the carbohydrate components linked to hydroxylysine of the collagenous protein²¹. The offspring of the diabetic mother are also exposed to hyperglycaemia and these infants are born with a variety of defects in development²². The ubiquitous presence of collagen throughout the body, its prime role as an inducer in early embryogenesis^{23,24}, and its susceptibility to structural changes have encouraged us to pursue further the regulation of synthesis and secretion of this molecule in the developing human organism.

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Spontaneous and induced cell-mediated reactivity to syngeneic cells

IN general, the immune system does not react with self-antigens to produce disease. Potential self-reactive clones are known to exist, however, and are thought to be controlled by thymic-derived suppressor lymphocytes (suppressor T cells). We have investigated the possibility that self-reactive clones of cells are expressed in B/W mice, and that such clones in other inbred mouse strains might become expressed after immunisation with specific antigens of the group A streptococcus. Our studies show that spleen cells from B/W mice respond spontaneously to syngeneic heart cells and to syngeneic kidney cells. In contrast, spleen cells from another inbred strain of mice, the BALB/c, do not respond spontaneously to syngeneic cells. A response to syngeneic cardiac determinants can, however, be induced in the BALB/c mouse by immunisation with cell membranes of group A streptococci.

Many observations, in both human and animal populations, suggest that at times self-reactivity (autoimmunity) does occur. One animal model of autoimmunity is the New Zealand Black/White hybrid mouse (B/W) (refs 1-3). In B/W mice, an imbalance of immune regulation precedes the onset of clinical autoimmune disease. Although the immunobiological events that result in autoimmune phenomena and the mechanisms by which these phenomena lead to pathological changes remain obscure, it has been proposed that the behaviour of T cells is critical to the development of autoimmunity⁴⁻⁹. T-cell suppressor function seems to be lost in the older B/W mouse, since exaggerated antibody responses to various antigens, such as type III pneumococcal polysaccharide and polyinosinic-polycytidylic acid, occur³⁻⁶. Further evidence for the loss of suppressor T-cell function is suggested by the decrease of concanavalin A-induced suppressor function in ageing mice¹⁰. Other T-cell functions also seem to be lost, since lymphoid cells from older B/W mice do not mount a graft against host response when transferred into allogeneic recipients.

Repeated exposure to antigens that cross react with self-determinants may induce an immune response that eventually results in autoimmunity. Repeated infections with group A streptococci are thought to be an essential factor in the pathogenesis of acute rheumatic fever¹¹. Immunological cross reactions between streptococcal and mammalian antigens have been demonstrated, and these cross reactions seem to be mediated by antibodies specific for antigens of the streptococcal cell surface¹²⁻¹⁵. In addition, there are several reports which suggest that cell-mediated cross reactions between streptococcal and mammalian antigens also may occur¹⁶⁻¹⁸.

In our experiments we used primary cultures of syngeneic

heart, liver or kidney prepared from neonatal B/W or BALB/c mice. The hearts, livers and kidneys from sex-matched neonatal mice were removed, minced in Hank's calcium and magnesium-free balanced salt solution, trypsinised, treated with mitomycin-C to block DNA synthesis, and resuspended in Eagle's minimal essential medium supplemented with 10% heat-inactivated foetal calf serum. These cell suspensions served as stimulator cells. Responder cells were obtained from the spleens of mice 2-12 months of age. Several groups of BALB/c mice were first immunised with suspensions of group A streptococcal cell membranes. Spleen cells were prepared as single-cell suspensions in the medium described. Responder spleen cells (1.8×10^5) and stimulator cells (1.8×10^4) from heart, kidney or liver were mixed and incubated for 6 d. On the sixth day, all cultures received $2 \mu\text{Ci}$ of tritiated thymidine, were incubated for an additional 24 h, and the amount of radioactive label incorporated into the acid-insoluble fraction of the cells was determined. Measurements of triplicate samples are reported as a stimulation index (SI) $\pm$ standard error of the mean (s.e.m.); the SI is the ratio of the mean d.p.m. of responder cells in stimulated culture minus the d.p.m. of stimulator cells alone to the mean d.p.m. of responder cells alone.

The response of the responder spleen cells after incubation with syngeneic or with allogeneic stimulator cells is shown in Table 1. B/W spleen cells responded to syngeneic heart and syngeneic kidney cells as well as to BALB/c heart cells, but did not respond to syngeneic liver cells. On the other hand, BALB/c spleen cells from unimmunised animals did not respond either to syngeneic heart or kidney cells or to heart cells from the histocompatible B/W mice. Sets of BALB/c mice were inoculated with group A streptococcal cell membranes, and their spleen cells were shown to respond to stimulation by streptococcal cell membranes. Spleen cells from these immunised BALB/c mice strongly reacted to both BALB/c heart cells and B/W heart cells, but did not respond to syngeneic kidney cells. The responsiveness of spleen cells from older B/W mice to syngeneic heart and syngeneic kidney cells persists, although they no longer respond to phytohaemagglutinin or concanavalin A (unpublished).

These findings demonstrate the existence of clones of cells in the B/W mouse that are spontaneously capable of responding to syngeneic cardiac and renal determinants. In addition, a similar response to syngeneic cardiac determinants can be induced in the BALB/c mouse by immunisation with cell membranes of group A streptococci. The response in the B/W mouse is present in young mice (1-2

Table 1 Response of spleen cells to syngeneic or allogeneic cell populations

Responder cells	Stimulator cells	SI $\pm$ s.e.m.
B/W	B/W Heart	38.7 $\pm$ 4.7
	B/W Kidney	9.5 $\pm$ 0.2
	B/W Liver	1.3 $\pm$ 0.4
	BALB/c Heart	92.2 $\pm$ 7.1
BALB/c (unimmunised)*	BALB/c Heart	0.6 $\pm$ 0.0
	BALB/c Kidney	0.0 $\pm$ 2.9%†
	B/W Heart	0.0 $\pm$ 1.3%†
BALB/c (immunised)‡	BALB/c Heart	82.0 $\pm$ 0.3
	BALB/c Kidney	1.8 $\pm$ 0.1
	B/W Heart	42.2 $\pm$ 1.2

*The unimmunised BALB/c responder cells cultured with streptococcal cell membranes yielded an SI of 1.2 ± 0.1 , whereas the immunised BALB/c responder cells yielded an SI of 25.7 ± 1.9 when cultured with the same cell membrane preparation.

†Responder cultures displayed less incorporation of ^3H -thymidine than control cultures of stimulator cells alone. SEM is reported as a percentage of the average of triplicate samples.

‡Immunised with group A type 14 streptococcal cell membranes.

months old) as well as in older mice (10–12 months old). These data are consistent with studies suggesting that the disturbance of immune regulation in the B/W mouse may include an early loss of suppressor T-cell function. A loss of suppressor T-cell function may well set the stage for the onset of autoimmune disease. Although these findings may shed some light on the pathogenesis of autoimmune disease in general, as well as that of rheumatic carditis, the observations that cell-mediated reactivity to syngeneic cardiac cells can occur spontaneously or can be induced by microbial antigens may be of broad immunobiological significance.

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Inhibition of platelet aggregation by a placental substance with prostacyclin-like activity

THE demonstration that certain arteries and veins have the ability to generate from prostaglandin (PG) endoperoxides an unstable substance called prostacyclin (PGI_2) (ref. 1), which will inhibit platelet aggregation^{2,3}, should lead to a reappraisal of the homeostatic mechanisms preventing thrombosis. It has been suggested that the endothelium of these blood vessels contains an enzyme which is capable of converting PG endoperoxides from circulating platelets into an unstable substance, prostacyclin. This inhibits platelet aggregation and so prevents the initial step of thrombus formation within the vessel⁴. Damage to the endothelium of the vessel wall may prevent production of the enzyme PGI_2 synthetase, and the subsequent reduction in the local synthesis of prostacyclin could facilitate platelet aggregation in the area of damage. Maintenance of an adequate circulation within the placenta is critical to foetal well-being. This circulation is one both of low pressure and velocity and the presence of a substance which has a potent inhibitory effect on platelet aggregation would seem desirable for its maintenance. We demonstrate here the ability of normal placental tissue to generate a labile substance which has the ability to inhibit ADP-induced platelet aggregation. The properties exhibited by this substance are suggestive of it being PGI_2 .

Venous blood was collected from the same subject who had not taken prostaglandin synthetase inhibitors for at least 2 weeks. Citrate solution 3.1% (9:1, v/v) was used as

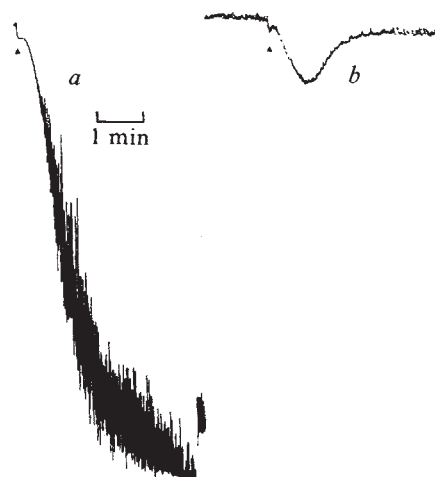


Fig. 1 Aggregation of platelet rich plasma (PRP) pre-incubated with placental tissue. PRP was prepared from citrated plasma by centrifugation at 2,000 r.p.m. for 10 min. Aggregation was induced by addition of ADP to a concentration of $5 \mu\text{M}$ ($\blacktriangle$). *a*, The control response of 0.9 ml PRP to ADP. *b*, The effect of pre-incubation of 0.9 ml PRP with 10 mg washed placental tissue for 20 min at 22°C before addition of ADP.

the anticoagulant and platelet rich plasma (PRP) and platelet poor plasma were prepared by centrifugation. Platelet aggregation was carried out as previously described⁵, using ADP in a final concentration of $5 \mu\text{M}$.

Pieces of placental tissue (10–40 mg) were taken at random from the maternal surface immediately after delivery. In all cases the pregnancies and deliveries were entirely normal. The placental pieces were washed several times to remove blood and kept in Krebs solution gassed with 95% O_2 :5% CO_2 until use.

Pre-incubation of PRP with placental tissue inhibited subsequent ADP-induced platelet aggregation (Fig. 1). Incubation of placental tissue in Tris buffer and the subsequent addition of an aliquot of the supernatant to PRP quantitatively inhibited ADP-induced platelet aggregation. Heating of this supernatant at 37°C for 10 min or boiling for 0.25 min abolished its anti-aggregatory properties (Fig. 2). Platelets aggregated by previous addition of ADP could be disaggregated by the addition of an aliquot of supernatant derived from incubation of placental tissue in Tris buffer (Fig. 3).

Pretreatment of placental tissue with indomethacin did not affect its anti-aggregatory properties when subsequently incubated with PRP, whereas pretreatment with tranlylcypromine abolished the anti-aggregatory activity (Fig. 4).

These experiments show that placental tissue can spontaneously generate a substance which inhibits platelet

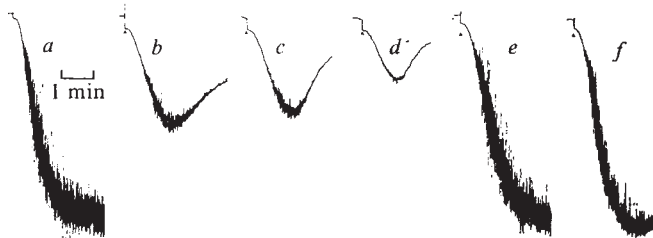


Fig. 2 Effects on platelet aggregation of supernatant from placental tissue incubated in buffer. 40 mg of placental tissue was incubated in 500 μl 0.05 M Tris, pH 7.5 for 20 min at 22°C . The supernatant was added in varying amounts. *a*, 0 μl ; *b*, 25 μl ; *c*, 50 μl and *d*, 100 μl —each added to 0.9 ml PRP and incubated for 1 min at 37°C before addition of ADP $5 \mu\text{M}$. The same experiments were carried out using 100 μl of supernatant which had been boiled for 0.25 min (*e*) or heated at 37°C for 10 min (*f*).

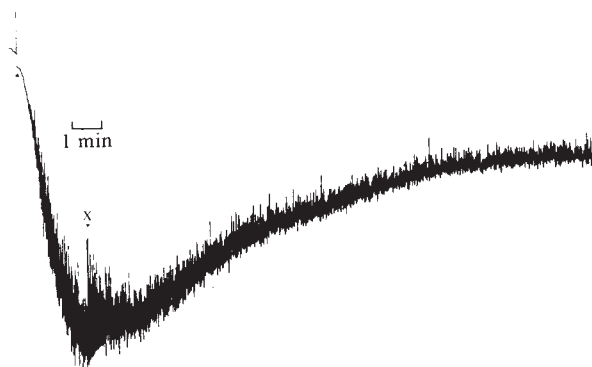


Fig. 3 Reversal of ADP-induced platelet aggregation by supernatant from placental tissue incubated in buffer. 0.9 ml PRP was aggregated by addition of ADP ($\blacktriangle$) and reversal of aggregation caused by addition at (X) of 100 μ l supernatant from 20 mg placental tissue incubated in 200 μ l 0.05 M Tris, pH 7.5 for 20 min at 22 $^{\circ}$ C.

aggregation. Addition of placental tissue itself (Fig. 1) or supernatant from tissue incubated in buffer at room temperature (Fig. 2) to PRP causes significant inhibition of ADP-induced platelet aggregation. Abolition of the anti-aggregatory activity by heating at 37 $^{\circ}$ C for 10 min or boiling for 0.25 min (Fig. 2) is in agreement with observations on the instability of prostacyclin³. Our findings that supernatant from placental tissue incubated in buffer can disperse preformed platelet aggregates (Fig. 3) is in agreement with the findings of other workers^{6,7}, confirming that this unstable substance affects platelet aggregation by means other than direct competition with thromboxane A₂ (ref. 8).

Pretreatment of the tissue with indomethacin, an inhibitor of the cyclo-oxygenase enzyme⁹ prevents formation in the tissue of PG endoperoxides and primary PGs known to inhibit platelet aggregation. Incubation of this indomethacin-treated tissue with PRP still inhibits platelet aggregation (Fig. 4). This suggests that the enzyme PGI₂ synthetase which may be present in the placenta is unaffected by indomethacin and can act on platelet endoperoxides to produce prostacyclin.

Pretreatment of placental tissue with tranilcypromine, a known inhibitor of PGI₂ synthetase⁴ does abolish the inhibi-

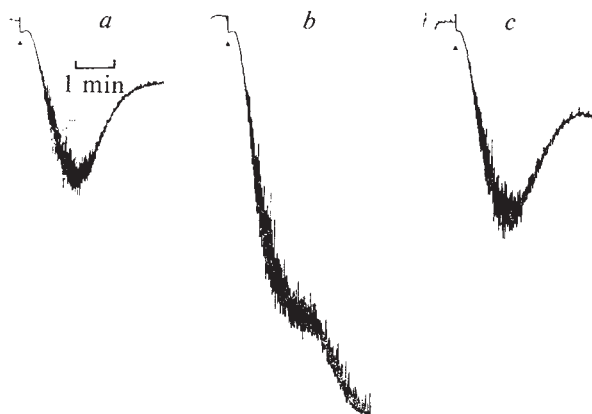


Fig. 4 Treatment of placental tissue with inhibitors of enzymes in the prostaglandin synthetic pathway. 20 mg of placental tissue was incubated for 20 min at 22 $^{\circ}$ C in a, 200 μ l 0.05 M Tris, pH 7.5; b, 200 μ l tranilcypromine (50 μ g ml⁻¹ in 0.05 M Tris, pH 7.5); c, 200 μ l indomethacin (1 μ g ml⁻¹ in 0.05 M Tris, pH 7.5). The tissue was removed, washed three times in Tris buffer before incubation with 0.9 ml PRP for 15 min at 22 $^{\circ}$ C and subsequent addition of ADP ($\blacktriangle$).

tion of platelet aggregation seen when placental tissue is incubated with PRP (Fig. 4).

Thus placental tissue when in contact with platelets seems to generate an unstable substance, inhibiting platelet aggregation and exhibiting the previously described properties of prostacyclin. The generation of this substance could be an important factor in the maintenance of placental circulation. Localisation and measurement of the PGI₂ synthetase capacity, which these experiments suggest might be present in the placenta, is the next step in understanding the homeostasis of the placental circulation. The decreased rate of platelet disaggregation in the plasma of patients suffering from placental insufficiency¹⁰ is suggestive of decreased prostacyclin levels or PGI₂ synthetase capacity.

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Prostacyclin is the major prostaglandin released from the isolated perfused rabbit and rat heart

PROSTACYCLIN (prostaglandin X, PGI₂), a newly discovered prostaglandin¹⁻³, is formed by microsomes from several organs, such as pig and rabbit aorta² and rat stomach⁴, and by fresh human arterial and venous tissue incubated with PGH₂ (ref. 5). As inhibitor of blood platelet aggregation, it is 30 times more potent than PGE₁ (ref. 2) and could thus help in preventing the formation of intra-arterial thrombi. It has been suggested¹ that the endoperoxides released by the platelets can be converted into prostacyclin by vascular tissue. Prostacyclin is chemically unstable in aqueous solution: it hydrolyses to 6-oxo-PGF_{1 α} (refs 3, 4; Fig. 1) which does not inhibit platelet aggregation². We report that isolated perfused rabbit and rat hearts produce a labile factor which strongly inhibits blood platelet aggregation; the release of this substance is abolished by indomethacin. Using a physicochemical method, we found 6-oxo-PGF_{1 α} in substantial amounts in the perfusates. These findings indicate that prostacyclin is the major prostaglandin released from the heart.

Hearts were perfused with a Krebs-Henseleit buffer solution according to Langendorff or as modified by De Deckere and Ten Hoor¹². The latter method is based on the observation that in the isolated perfused heart a small part (2-5%) of the perfusion fluid reaches the surface of the heart through the interstitial space and the lymphatics. This smaller flow is separated from the main flow by tying off the veins of the right and left atrium and cannulating the pulmonary artery. Most of the perfusion fluid is ejected by the right ventricle through the cannula (Q_{rv}), a small amount passes the interstitium and drips from the heart (Q_i). As the flow rate of Q_i is small, the concentrations of substances released into Q_i are relatively high.

Prostacyclin-like activity was determined direct in Q_i by measuring the inhibition of ADP-induced rat platelet aggregation (Fig. 2)⁶. The biological activity was standardised against PGE₁. As rat platelets are insensitive to PGD₂ and PGE₂,

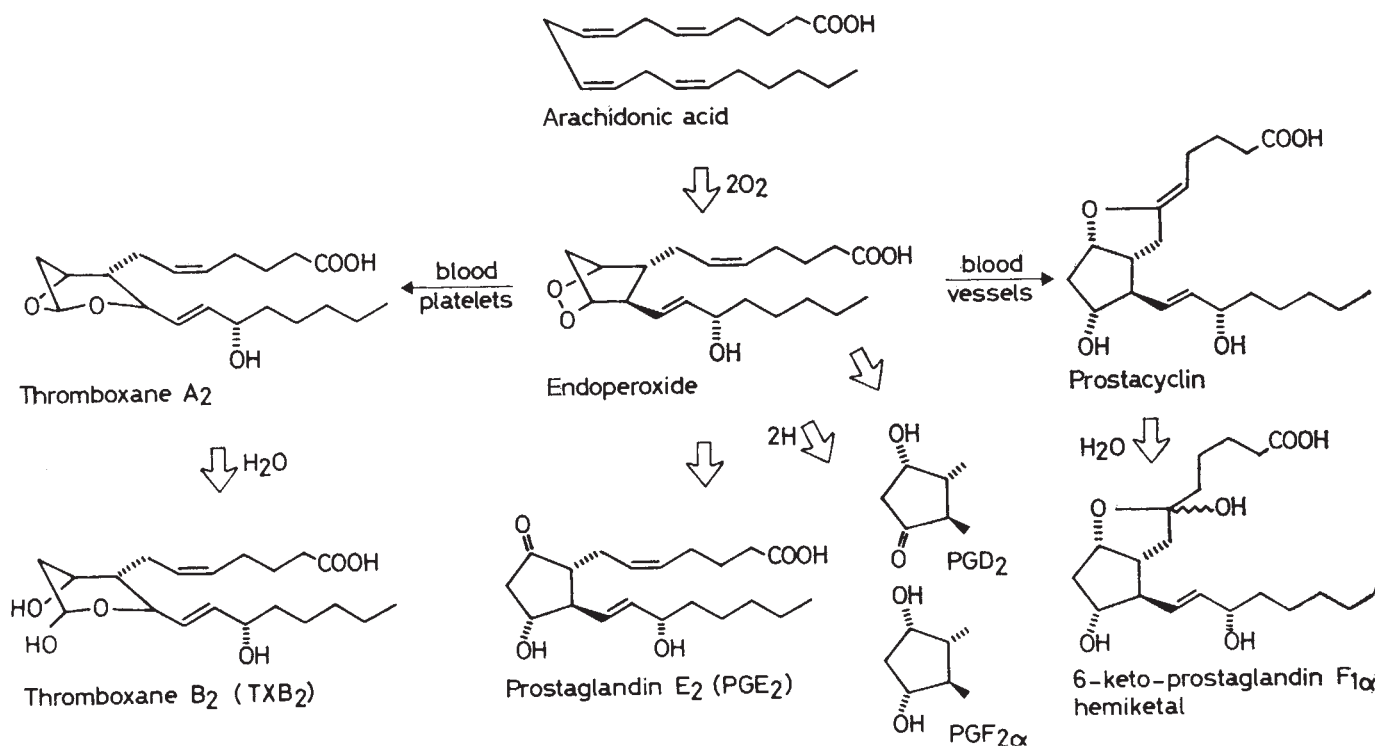


Fig. 1 Routes in the metabolism of prostaglandin-endoperoxide.

interference from 'stable' prostaglandins in Q_1 cannot be expected. PGE₂-like activity was assayed on the isolated gerbil colon⁷ in ethyl acetate extracts of acidified perfusates. Antagonists were used as described previously⁸. PGE₂, PGD₂, PGF_{2α}, TXB₂ (thromboxane B₂) and 6-oxo-PGF_{1α} (Fig. 1) were extracted, purified by thin-layer chromatography as their methoximated pentafluorobenzyl esters and, after silylation, determined by gas chromatography with electron-capture detection⁹ (Table 1). Figure 3 gives examples of the results of the determination of PGE₂, PGF_{2α} and 6-oxo-PGF_{1α}.

The isolated perfused rabbit heart released at least twice as much 6-oxo-PGF_{1α} as PGE₂, the amount (8–12 ng per min per g dry weight) being more than that of all other prostaglandins together (Table 2). The results of the bioassay on the gerbil colon and the gas-chromatographic determinations of PGE₂

correlate reasonably well. Since 6-oxo-PGF_{1α} is the stable hydrolysis product from prostacyclin, it is most probably prostacyclin that was released from the isolated rabbit heart. Freshly collected Q_1 showed a distinct inhibition of platelet aggregation (Fig. 2 and Table 3), an ability which disappeared after incubating Q_1 for 10–15 min at 37 °C. Perfusion of the heart with indomethacin (1 μg ml⁻¹) completely abolished the anti-aggregatory activity of the perfusate; anoxia for 6 min increased this activity only moderately. Because of the much greater dilution, no inhibition of platelet aggregation could be detected with Q_{IV} , although in this perfusate 6-oxo-PGF_{1α} was present according to gas chromatography.

Perfused rat hearts also released 6-oxo-PGF_{1α} (Table 3) as the major prostaglandin, but anoxia did not increase prostaglandin output. In ten rat hearts Q_1 was collected for 5 min

Table 1 R_f values of the methoximated (MO) pentafluorobenzyl (PFB) esters after TLC and retention times (R_t) of the trimethylsilylated (TMS) derivatives during GLC

Compound*	%†	R_f (MO·PFB)	R_t ‡ (MO·PFB·TMS) (min)	Compound*	%†	R_f (MO·PFB)	R_t ‡ (MO·PFB·TMS) (min)
PGD ₂	90	0.69	16.0	Thromboxane B ₂ (TXB ₂)	43	0.46	19.8
	10	0.57	14.7		57	0.38	19.8
PGE ₂	74	0.54	17.5	6-oxo-PGF _{1α}	58	0.37	22.0
	26	0.46	15.2		42	0.31	22.2
ω-nor-PGE ₂	68	0.53	14.2	PGF _{2α}	—	0.28	15.2
	32	0.45	12.3	PGF _{1α}	—	0.25	17.8

100 ng ω-nor-PGE₂ (ref. 10) and 100 ng PGF_{1α} in 1 ml methanol were added to the perfusates as internal standards. (For the experiment of Table 2, the two standards were added after extraction.) The solution was then acidified with HCl (6 mol l⁻¹), drop by drop, until pH 4.0. The perfusates were extracted twice with equal volumes of ethyl acetate. The extract was taken to dryness *in vacuo* at 30 °C and the residue rinsed with 2–6 ml methanol into a small test tube. The solvent was removed with nitrogen, and 0.2 ml 2% methoxyamine HCl in pyridine was added. After 16 h at 20 °C, the greater part of the pyridine was removed with nitrogen; 0.5 ml H₂O was added and the prostaglandins were extracted with 2 × 2 ml ether. To the residue of this extraction, 30 μl pentafluorobenzylbromide was added followed by 60 μl 10% diisopropylethylamine in acetonitrile⁹. After standing for 1 h at 20 °C, the solvents were again removed with a nitrogen stream. The residue was applied as a 2-cm band on a 0.25-mm thick silica gel Fertigplatte (Merck) and developed with chloroform-methanol (90:6, v/v). The appropriate bands were scraped off the plate and eluted with 1–2 ml ether-methanol (2:1, v/v). The eluates were evaporated, the residue was silylated with 25 μl *bis*-(trimethylsilyl)-trifluoroacetamide-pyridine (1:1, v/v) for 1 h at 20 °C and excess reagent removed with nitrogen. Between 0.5 and 5 ng of the PFB esters was injected for gas chromatography, giving sufficiently large peaks with the electron-capture detector.

*Authentic prostaglandins used as references and standards were obtained by biosynthesis from the corresponding unsaturated fatty acids or by incubating PGH₂ with blood platelets (TXB₂) or with sheep aorta microsomes (6-oxo-PGF_{1α}).

†The methoximes were present as two isomers (*syn* and *anti*) in the ratio as indicated. PGF_{2α} and PGF_{1α} cannot give a methoxime.

‡2% SE 30, $l = 150$ cm, $d = 0.2$ cm, at 230 °C; carrier gas: argon–10% methane, 25 ml min⁻¹. A Hewlett-Packard 5700 A gas chromatograph with a linear electron capture detector 18713 A was used.

Table 2 Prostaglandin release (ng per min per g dry weight) from the isolated, perfused rabbit heart after anoxia

Group	Gerbil colon	Gas chromatography				
	PGE ₂ -like activity	PGE ₂	PGD ₂	PGF _{2α}	TXB ₂	6-oxo-PGF _{1α}
1 (n=8)	4.7±0.6	3.5	0.9	1.8	2.2	9.3
2 (n=8)	3.3±0.5	3.9	1.2	1.8	2.1	9.9

In two separate experiments, eight rabbit hearts (about 0.7 g dry weight) were perfused according to Langendorff (for details, see Table 3). Fifteen minutes after starting the perfusion, the hearts were perfused with an oxygen-free buffer for 3 min. The buffer was then reoxygenated and the perfusion fluid collected for 10 min to give about 400 ml perfusate per heart. This fluid, after acidification, was extracted with ethyl acetate (Table 1). Aliquots of the extracts were tested individually for PGE₂-like activity; PGE₂ served as standard. The other prostaglandins found in the perfusate have relatively little activity on the gerbil colon. The remaining parts of the samples were pooled in the two groups of eight and the five prostaglandins determined in triplicate.

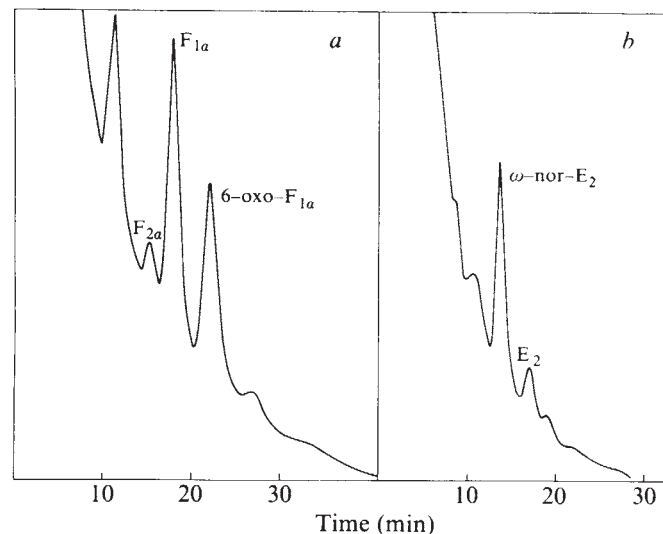


Fig. 3 Gas-chromatographic results of the determination of PGF_{2α} (a), 6-oxo-PGF_{1α} (a) and PGE₂ (b) in rabbit heart perfusates. The methoxime-pentafluorobenzylester-trimethylsilyl-ether derivatives were used for the electron-capture detection. PGF_{1α} (100 ng) and ω-nor-PGE₂ (100 ng) had been added as internal standards. Attenuation × 128. For experimental details, see Table 1.

(from 20 to 25 min after starting the perfusion). The prostacyclin-like activity was determined by the platelet aggregation test, the prostacyclin production being 3.3 ± 0.4 ng per min per g dry weight. In four of ten hearts, the prostacyclin release, observed for 1 h at 15-min intervals, was nearly constant. As the release of prostacyclin into Q_i and Q_r is about the same, the total release of prostacyclin from the rat heart can be estimated from the amount released into Q_i.

Our results show that prostacyclin is the major prostaglandin released from perfused rabbit and rat hearts. The negligible amount of thromboxane B₂ found in the perfusion fluid renders it likely that there are hardly any blood platelets present in the isolated perfused heart. This indicates that the heart can synthesise prostacyclin direct from arachidonic acid. We also found that the amount of prostacyclin released from the heart is almost constant during perfusion for more than 1 h, and that it is abolished by infusion of indomethacin ($1 \mu\text{g ml}^{-1}$). Moncada and co-workers found that in rabbit and pig aortas¹ and in human arteries and veins⁵ prostacyclin is almost exclusively synthesised from prostaglandin endoperoxides. They suggest that *in vivo* endoperoxides originating from blood platelets, in active contact with the endothelial layer, are converted into prostacyclin. Our results show that the rabbit and rat heart can synthesise prostacyclin in the absence of blood platelets.

The physiological consequences of these findings cannot yet

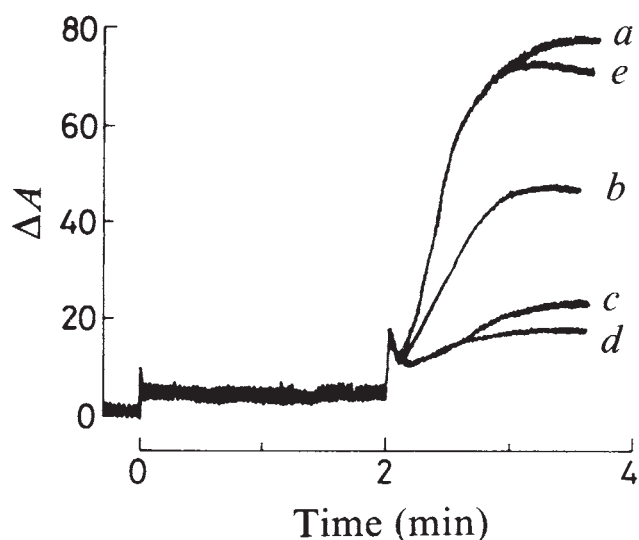


Fig. 2 Inhibition of ADP-induced rat platelet aggregation by freshly collected interstitial effluent Q_i from the isolated perfused heart. Changes in absorbance (ΔA) of rat platelet-rich plasma (PRP; final volume 3 ml) in a modified Vitatron photometer at 37 °C. At $t = 0$, 0.2 ml buffer solution (Krebs-Henseleit) (a), 0.2 ml buffer solution with a PGE₁ concentration of 60 (b) or 120 ng ml⁻¹ (c), 0.2 ml of Q_i (d) or 0.2 ml of Q_i heated at 37 °C for 15 min (e) was incubated with PRP for 2 min ADP (final concentration 94 ng ml⁻¹) was then added to induce aggregation. Tracings have been superimposed. As prostacyclin is assumed to be 30 times more potent than PGE₁ (ref. 2), it can be calculated that in this case Q_i contains 4.5 ng ml⁻¹ prostacyclin. Tracing e shows that heating of Q_i abolishes its anti-aggregatory activity.

Table 3 Prostacyclin and prostaglandin release (ng per min per g dry weight) from the isolated perfused rabbit and rat heart

	Perfusate	Rabbit			Rat			
		Prostacyclin	6-oxo-PGF _{1α}	PGE ₂	PGD ₂	PGF _{2α}	TXB ₂	6-oxo-PGF _{1α}
Before anoxia	Q _{rv}	—	2.7	2.9	<0.5	0.5	0.6	2.2
	Q _i	4.2	4.0	0.5	<1	0.4	0.3	3.1
Following anoxia	Q _{rv}	—	2.2	1.2	n.d.*	0.6	n.d.*	1.8
	Q _i	6.2	8.7	0.7	n.d.*	0.2	n.d.*	1.7

Hearts were perfused with a Krebs-Henseleit buffer solution, gassed with O₂-CO₂ (95:5) and supplemented with glucose (11.1 mmol l⁻¹) and EDTA (0.5 mmol l⁻¹), by the modified Langendorff technique of De Deckere and Ten Hoor¹². Perfusion pressure was 60 mmHg for rabbit hearts, 80 mmHg for rat hearts; stimulation frequencies were 250 and 360 min⁻¹. Twenty minutes after starting the perfusion Q_{rv} and Q_i were collected for 20 min from two rabbit hearts (about 1 g dry weight per heart; hearts were dried at 80 °C for 24 h; dry weight was about 20% of wet weight) and four rat hearts (about 0.2 g dry weight per heart) to give a total of 1400 ml Q_{rv} and 22 ml Q_i from the rabbit hearts and 1,200 ml Q_{rv} and 30 ml Q_i from the rat hearts. Next, the rabbit hearts were perfused with a buffer, gassed with N₂-CO₂ (95:5) for 6 min, the rat hearts for 5 min. Following this anoxic period, the perfusion fluid was reoxygenated and Q_{rv} (rabbit 850 ml, rat 600 ml) and Q_i (rabbit 40 ml, rat 20 ml) were collected for 10 min. Prostacyclin-like activity was determined direct in fresh Q_i samples collected on ice under N₂-CO₂ (95:5), using the blood platelet aggregation assay⁶ and assuming an activity of 30 times that of PGE₁ (ref. 2). Prostaglandins were determined by gas chromatography.

*n.d., Not determined.

be estimated. The continuous production of prostacyclin by the heart and the anti-aggregatory and vasodilatory activity suggest that the main function of prostacyclin production is to protect the coronary circulation against the formation of blood platelet aggregates, thus preventing the occlusion of small branches of the vulnerable coronary tree. If the present results are extrapolated to the human heart, prostacyclin could be a key substance with respect to the origin¹¹ as well as the prevention of coronary diseases.

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Transitory expression of Thy-1 antigen in skeletal muscle development

A CELL surface differentiation antigen known as Thy-1 (formerly called theta¹) is expressed during development of brain and thymocytes in both rat and mouse (see ref. 2 for review). Thy-1 is present in brain and on thymocytes throughout adult life, as well as on a subpopulation of peripheral lymphocytes. Both rat and mouse fibroblasts, and some fibroblastic cell lines,

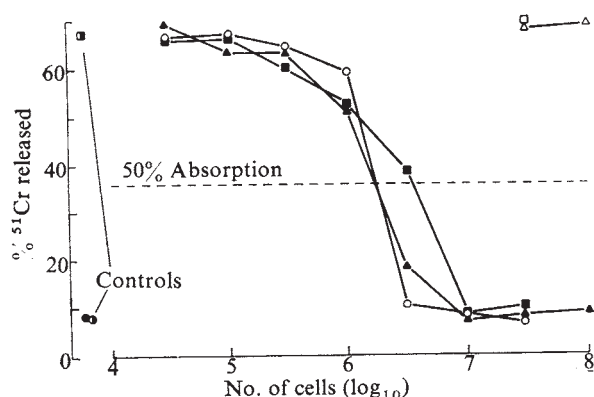


Fig. 1 Exponentially growing cells were harvested in HEPES buffered medium containing 2% FCS (ABM/FCS). L6 and M3A were loosened from the dishes before cell collection by incubation at 37 °C for 5 min in phosphate buffered saline (PBS) lacking Ca²⁺ and Mg²⁺ and containing 2 mM EDTA. Graded numbers of cells were added to ATS (0.2 ml of a 1:80 dilution in HBM/FCS) for 45 min at 4 °C, then removed by centrifugation. Residual antibody was assayed by adding 0.1 ml of absorbed ATS to ⁵¹Cr-labelled SIA target cells (2 × 10⁶) in 0.2 ml HBM/FCS containing fresh rat serum complement at a dilution of 1:8. After 45 min at 37 °C, 2 ml HBM/FCS was added, the tubes were centrifuged for 10 min at 475 g, and supernates and pellets were counted separately. %⁵¹Cr release was calculated as follows

$$\frac{\text{c.p.m. in supernate}}{\text{c.p.m. in supernate} + \text{c.p.m. in pellet}} \times 100$$

Cell lines used for absorption were: ○, SIA; ▲, C58[NT]D; ■, L6; △, S194; □, M3A. Controls were ⁵¹Cr SIA target cells: ●, alone (spontaneous lysis); ○, with rat complement; □, with rat complement + unabsorbed ATS at 1:80.

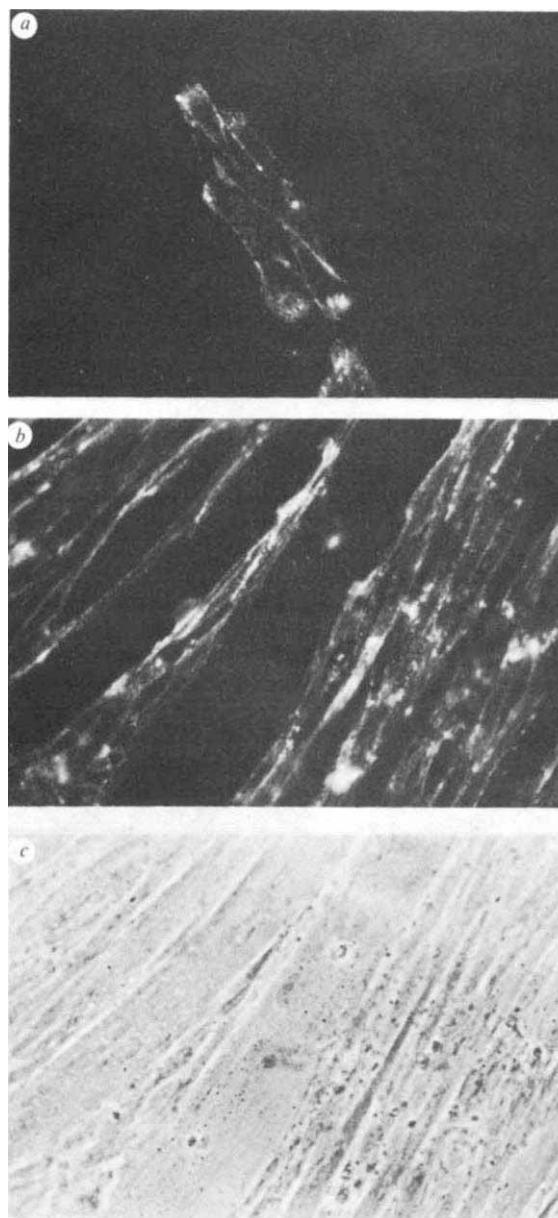


Fig. 2 Indirect immunofluorescence assays were performed on living cells grown on coverslips. After washing with warm HBM containing 5% FCS, ATS was added at a 1:50 dilution for 30 min at room temperature. The cells were washed twice more, fluorescein conjugated IgG fraction of goat anti-rabbit IgG (Cappel Laboratories) was added at a 1:50 dilution for 30 min in the dark at room temperature. After a final two washes in HBM/5% FCS, and a brief rinse in PBS, the coverslip was inverted over a drop of glycerol and PBS (9:1) and examined at 440 nm by a Zeiss fluorescent microscope with epi-illumination. *a*, L6 myoblasts (indirect immunofluorescence with ATS), 3 d culture (×875); *b* and *c*, (fluorescence with ATS and phase contrast, respectively) L6 myoblasts and multinucleate myotubes 13 d cultures (×875).

express Thy-1 *in vitro*³ and mouse Thy-1 has been reported on epidermal cells (see ref. 2 for review) and a mammary tumour⁴. The Thy-1 antigen has been reported lacking in other adult tissues, including muscle¹. We present here evidence for an antigen with the serological specificity of Thy-1 on rat myogenic cell lines and embryonic rat muscle differentiating *in vitro*. The presence of the antigen was transitory, being expressed on myoblasts and newly developed myotubes, but disappearing as myotubes differentiated.

Rabbit anti-rat-thymocyte serum (ATS) was raised by injecting 10⁶ Lewis rat thymocytes, intraperitoneally weekly together with *B. pertussis*, and intradermally every 6-12 weeks with complete Freund's adjuvant. Complement dependent

cytotoxicity was titrated on rat thymocytes. Sera of high cytotoxic titres were pooled, heated at 56 °C and absorbed sequentially at 4 °C with rat red blood cells, liver and kidney. A similarly raised and absorbed antiserum was reported to have specificity for purified Thy-1 glycoprotein (see ref. 5). Full details of our pooled antiserum's specificity will be published elsewhere (J.F.L. and V.A.L., in preparation). Its Thy-1 specificity was established by the following criteria: (1) complement dependent cytotoxicity for rat and mouse Thy-1 bearing lymphocytes and lymphomas but not for mouse myelomas; (2) absorption of cytotoxicity against a mouse lymphoma by Thy-1 positive mouse lymphomas, but not by Thy-1 negative variants⁶; (3), absorption by rat brain and thymus, but not by liver, kidney, adult muscle or red blood cells; and (4), precipitation of the 25,000 molecular weight glycoprotein T25 (bearing Thy-1 allospecificity⁷) from ¹²⁵I-labelled proteins solubilised from rat and mouse thymocyte surfaces.

Thy-1 was assayed by quantitative absorption of cytotoxicity⁸ and by indirect immunofluorescence. In assaying for residual antibody after absorption, mouse rather than rat Thy-1 bearing lymphomas were used as cytotoxic targets to ensure detection

of a Thy-1 specificity since T25 was the only detectable molecule precipitated from mouse thymocytes by ATS.

Cell lines assayed included: cloned rat myogenic lines, L6 (derived by repeated subculture of foetal limb muscle⁹) and M3A (a non-fusing variant of L6 (ref. 10)), a W/Fu rat thymoma (C58[NT]D (ref. 11)), a BALB/c mouse lymphoma bearing Thy-1 (SIA (ref. 12)) and a BALB/c myeloma (S194 (ref. 12)). All cells grew in suspension in Falcon Petri dishes in Dulbecco's modified Eagles medium (DMEM) with 10% foetal calf serum (FCS), except L6 and M3A which grew attached to Falcon tissue culture dishes and were harvested with 2 mM EDTA for absorption assays.

Figure 1 illustrates the results of absorption assays. Cytotoxicity of ATS for the Thy-1 bearing target SIA was 50% absorbed by 1.5×10^6 rat or mouse thymoma cells. This is approximately twice the absorptive capacity of rat thymocytes. Fifty per cent cytotoxicity was absorbed by 3.0×10^6 L6 myoblasts. Thus the absorptive capacity of each L6 cell approximated that of a thymocyte. Estimation of cell surface area allowed calculation of the approximate antigen density represented by the absorption capacities (see ref. 8). When rounded up after harvesting the

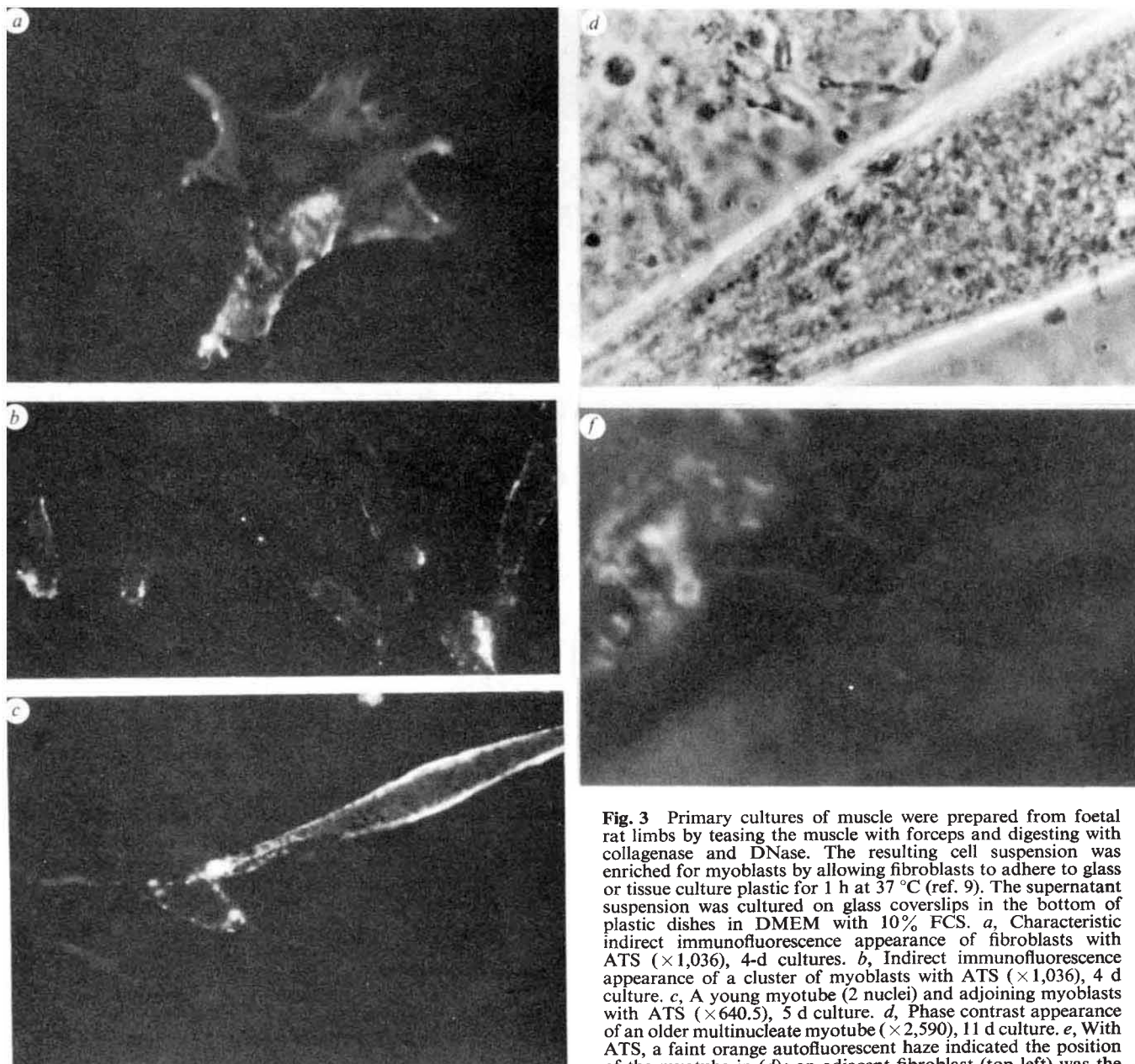


Fig. 3 Primary cultures of muscle were prepared from foetal rat limbs by teasing the muscle with forceps and digesting with collagenase and DNase. The resulting cell suspension was enriched for myoblasts by allowing fibroblasts to adhere to glass or tissue culture plastic for 1 h at 37 °C (ref. 9). The supernatant suspension was cultured on glass coverslips in the bottom of plastic dishes in DMEM with 10% FCS. *a*, Characteristic indirect immunofluorescence appearance of fibroblasts with ATS ($\times 1,036$), 4-d cultures. *b*, Indirect immunofluorescence appearance of a cluster of myoblasts with ATS ($\times 1,036$), 4 d culture. *c*, A young myotube (2 nuclei) and adjoining myoblasts with ATS ($\times 640.5$), 5 d culture. *d*, Phase contrast appearance of an older multinucleate myotube ($\times 2,590$), 11 d culture. *e*, With ATS, a faint orange autofluorescent haze indicated the position of the myotube in (*d*); an adjacent fibroblast (top left) was the typical green of fluorescein.

antigen density of the L6 myoblast was approximately one-third that of a thymocyte. M3A, its non-fusing variant, and S194, a myeloma, did not absorb ATS activity, indicating lack of Thy-1 antigen (less than 0.5–1% of thymocytes).

As L6 cells mature, myoblasts fuse forming multinucleate myotubes which bear nicotinic acetylcholine receptors, are contractile, contain enzymes characteristic of mature muscle and are capable of forming functional cholinergic synapses when co-cultured with appropriate neurons¹³. We noticed that older L6 cultures containing many large myotubes (14 d after plating) absorbed less ATS cytotoxicity than cultures of recently plated myoblasts. The distribution of antigen on L6 was examined by indirect immunofluorescence with ATS (Fig. 2). With fine focus adjustment uniform speckling was observed over the surface of all myoblasts (Fig. 2a). L6 cells incubated with either ATS previously absorbed with rat brain or with fluoresceinated anti-rabbit IgG alone, were not fluorescent. In older cultures large, multinucleate L6 myotubes lacked surface fluorescence, in contrast to surrounding myoblasts (Fig. 2b and c).

Immunofluorescence studies of Thy-1 in cultured foetal muscle are complicated by the presence of fibroblasts, which express Thy-1 (ref. 3). The modified spindle shape of a typical myoblast (Fig. 3b) distinguishes it from flattened irregular-shaped fibroblasts (Fig. 3a). In early primary cultures all cells fluoresced with ATS, the pattern on primary myoblasts being similar to L6 myoblasts. The density of fluorescent speckles on individual primary myoblasts varied considerably, however. The brightest myoblasts resembled L6 in both pattern and intensity of fluorescence; others had fewer speckles distributed randomly over the cell surface. The surface fluorescence of newly formed myotubes, with just a few nuclei, appeared more dense and brighter than that of adjacent myoblasts (Fig. 3c). Fluorescence on myoblasts, young myotubes and fibroblasts was absorbed by rat brain, but not by adult muscle or liver. Mature multinucleate myotubes (Fig. 3d) were virtually invisible under blue light (Fig. 3e).

Further evidence for the Thy-1 specificity of immunofluorescence on cultured muscle cells includes the following: (1) Rat brain cell lines which were negative for Thy-1 by absorption assay failed to fluoresce with ATS while those positive by absorption were also positive by immunofluorescence (J.F.L. and V.A.L., in preparation). (2) The positive immunofluorescence of mouse primary muscle cultures with ATS, was absorbed by a Thy-1 positive lymphoma, but not by its Thy-1 negative variant⁶. (3) Primary rat myoblasts and fibroblasts were positive by indirect immunofluorescence using an antiserum raised against T25 (ref. 7), a glycoprotein bearing Thy-1 specificities.

Accompanying *in vitro* differentiation of foetal muscle and myogenic cell lines there was apparent loss of Thy-1 which was expressed on myoblasts but was not detectable by immunofluorescence on mature myotubes. This observation is consistent with lack of Thy-1 in adult muscle. The transitory appearance of Thy-1 on myogenic cells at the time of fusion and its absence on a non-fusing variant of the L6 myoblast line suggests that this antigen may be functional in normal muscle development. Glycoproteins bearing Thy-1 antigenic determinants have been purified from rat brain and thymus². The chemical nature of Thy-1 antigen expressed in developing muscle is yet to be established.

The occurrence on myoblasts and immature myotubes of an antigen characteristic of thymocytes represents another unexplained association between muscle and thymus. Others include: (1) the presence of striated muscle ('myoid') cells as a constituent of normal mammalian, avian and reptilian thymuses¹⁴; (2) the presence in rat thymus of nicotinic acetylcholine receptors characteristic of muscle¹⁵; (3) the concurrence of pathological lesions in thymus and muscle in at least two spontaneous disease processes—myasthenia gravis and murine muscular dystrophy¹⁶; (4) the reported *in vivo* effect on neuromuscular transmission of thymopoietin, a putative thymic

hormone which induces the expression of Thy-1 on cultured prothymocytes^{17,18}. The possible function of an antigen shared by developing muscle and thymocytes, T lymphocytes and brain is open to speculation.

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Relationship between Na:K and Na:Na exchange by the sodium pump of skeletal muscle

THE sodium pump can catalyse several modes of cation exchange (for example Na:K, Na:Na, and K:K), which have been characterised mainly in human red blood cells^{1–3}, squid giant axon^{4–6}, and frog skeletal muscle^{7–9}. In all three tissues it seems that intracellular ADP levels determine the extent to which the pump can engage in Na:Na exchange^{2,6,9,10}. Yet, despite its obvious kinetic implications, the basic question of how the 'normal' Na:K exchange is affected by the appearance of ADP-induced Na:Na exchange, has not been answered. Here we present evidence that, in frog skeletal muscle, elevated intracellular ADP levels have little effect on pre-existing electrogenic Na:K exchange, but cause Na:Na exchange to appear in addition to ongoing Na:K exchange.

Frog sartorius muscles, which had been loaded with sodium and depleted of potassium by storage for approximately 48 h at 3 °C, were used. Previous work has shown that 2, 4-dinitrofluorobenzene (DNFB) is a metabolic inhibitor¹¹, raises intracellular ADP levels^{9,12}, and induces sodium pump-mediated Na:Na exchange in this preparation⁹. The experiments that follow show that DNFB does not appreciably alter Na:K exchange.

Strophanthidin-sensitive K influx was determined in muscles treated with DNFB to raise their ADP levels, and in controls. Figure 1 shows that elevation of ADP levels had only a small effect on K influx (there was little effect on either V_{max} or K_m). If Na:Na exchange, which we know to arise in such muscles (see ref. 9 and Fig. 2), actually took the place of Na:K exchange, then strophanthidin sensitive K influx should have been inhibited by induction of Na:Na exchange. There is no indication that this happened.

The sodium pump operates in markedly electrogenic fashion in sodium-loaded frog sartorius muscle fibres¹⁴. Thus, strophanthidin-sensitive membrane current can be used as another

index of the extent of Na:K exchange, assuming that Na:Na exchange is electroneutral^{1, 5, 9}. Table 1 lists measurements of membrane potential in a second series of sodium loaded muscles, bathed in 10 mM K low-chloride Ringer's. DNFB did not significantly alter ouabain-sensitive membrane potential or membrane resistance. We conclude, therefore, that elevation of ADP did not alter the current generated by the sodium pump, further evidence against enhancement of Na:Na exchange at the expense of Na:K exchange.

The above argument rests on a comparison made between separate batches of muscles, those used for K influx measurements compared with those used for Na influx measurements. A direct quantitative comparison of the two exchange mechanisms is therefore of value. Strophanthidin sensitive ²²Na influx (an index of pump-mediated Na:Na exchange) and strophanthidin-sensitive ⁴²K influx (an index of Na:K exchange) were measured in the same muscles. Figure 2 shows values for Na and K influxes in DNFB-treated and untreated muscles, with and without strophanthidin present. Strophanthidin-sensitive differences are plotted on the right. There is marked stimulation of Na influx (Na:Na exchange) but only slight inhibition of K influx (Na:K exchange). Assuming the pump's exchange rate to be 3:2 per turnover for normal Na:K exchange, and 3:3 when the pump is engaged in Na:Na exchange, then the observed K influx decrease of 1.6 $\mu\text{mol g}^{-1} \text{h}^{-1}$ should result in the induction of just under 2.4 $\mu\text{mol g}^{-1} \text{h}^{-1}$ Na influx by direct substitution. Since Na influx increased by 8.5 $\mu\text{mol g}^{-1}$

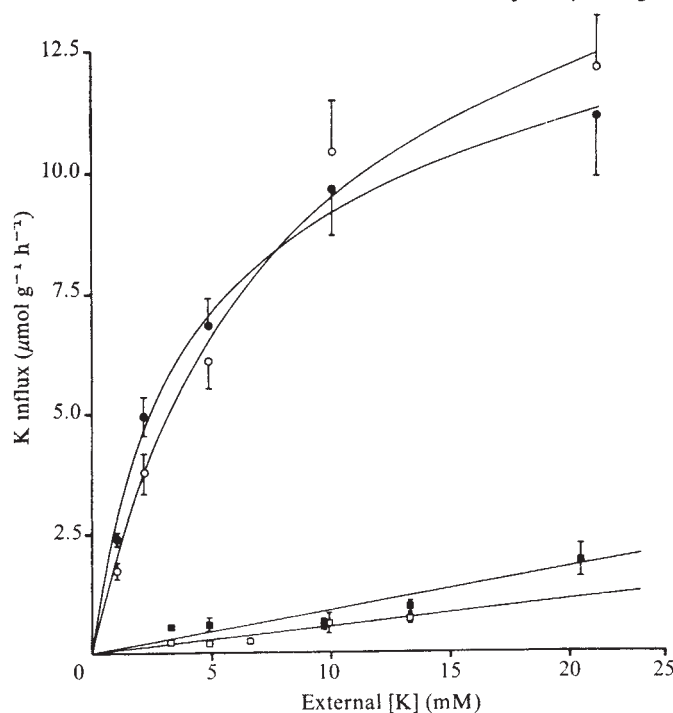


Fig. 1 Unidirectional K influx as a function of external [K] in DNFB-treated and untreated muscles. Values are means $\pm$ s.e.m. $\square$, $\blacksquare$, muscles exposed to 10^{-4} M strophanthidin; $\circ$, $\bullet$, muscles not exposed to strophanthidin. Open symbols refer to muscles treated for 30 min with 0.38 mM DNFB; closed symbols refer to untreated muscles. The data obtained in the presence of strophanthidin were fitted by straight lines; the strophanthidin sensitive fluxes were fitted by rectangular hyperbolae. Muscles were stored in K-free Ringer's solution in the cold for approximately 48 h before the experiment. All solutions contained 5 mM Ba (hence the low level of strophanthidin-insensitive K influx)¹³, and 10^{-7} g ml⁻¹ tetrodotoxin to prevent twitching. K was elevated by substitution for Na, which was 115 mM in K-free Ringer's solution. Muscles were exposed to ⁴²K for 25 min (K influx is essentially linear for at least 1 h)⁷ and total intracellular radioactivity was determined by back extrapolation to zero time of the linear portion of the washout curve. Parameters were: maximal influx, $10.92 \pm 1.03 \mu\text{mol g}^{-1} \text{h}^{-1}$, and K_m , 3.19 ± 0.73 mM, for control muscles; and maximal influx, $14.94 \pm 1.70 \mu\text{mol g}^{-1} \text{h}^{-1}$, and K_m , 6.76 ± 1.49 mM, for DNFB-treated muscles. There was a marginally significant effect of DNFB on both parameters ($0.05 > P > 0.02$).

Table 1 Effect of DNFB on the ouabain-sensitive potential, and membrane resistance, of frog sartorius muscles

	Membrane potential (mV)	
	Control	DNFB-treated
Without ouabain	-67.7 ± 1.6 (5)	-72.5 ± 2.0 (5)
With ouabain	-49.3 ± 2.1 (5)	-56.8 ± 2.2 (5)
Ouabain-sensitive	18.4 ± 2.6	15.7 ± 2.9
	Membrane resistance ($\text{k}\Omega \text{cm}^2$)	
	Control	DNFB-treated
Without ouabain	4.9 ± 0.5 (4)	3.8 ± 0.4 (4)
With ouabain	4.8 ± 0.6 (4)	4.3 ± 0.6 (4)

Values are means $\pm$ s.e.m. with the number of muscles indicated in parentheses. The difference in ouabain-sensitive potential between control and DNFB-treated muscles was not significant ($P > 0.2$), nor was the effect of DNFB and/or ouabain on membrane resistance ($P > 0.1$). Muscles were loaded with sodium by storage for 48 h at 3 °C in K-free Ringer's solution. Apart from this loading solution, all other solutions were low in chloride (10 mM) with isethionate used as a substitute. Low-chloride solutions were used to increase membrane resistance and to prevent the ouabain-induced membrane conductance increase described by Geduldig¹⁶. Washout of internal Cl from the muscle was by 3 h storage at 3 °C in K-free, low-Cl Ringer's. Muscles were next equilibrated with K by storage, still at 3 °C, in 10 mM K, low-Cl Ringer's for 45 min. Muscles from 18 frogs were then exposed to 10 mM K, low-Cl Ringer's at room temperature, and muscles from another 18 frogs to a similar solution containing 0.38 mM DNFB. Of each muscle pair from the same frog, one member was exposed to 10^{-4} M ouabain as well. Membrane potential measurements were begun after 20 min and continued for another 20 min. (Since the DNFB effect peaks after 30 min exposure⁹, this procedure bracketed the period of maximum Na:Na exchange.) Conventional 3M KCl-filled microelectrodes of approximately 3 mV tip potential and 20 M Ω resistance were used. Two microelectrodes were re-used to determine membrane resistance—one to pass current and the other to record steady-state membrane potential deflection. Input resistance and space constants were obtained from a semi-log plot of potential deflection against electrode separation. Membrane resistance could then be calculated by one-dimensional cable analysis, assuming internal resistance to be 169 Ωcm (ref. 16).

h^{-1} it seems that a substantial amount of 'new' Na:Na exchange must have occurred in addition to ongoing Na:K exchange unless there is a vast difference in turnover rate between the two modes. The work of Garrahan and Glynn in red blood cells^{1,2}, as well as our own in frog muscle (unpublished) suggests that the turnover rate in the two modes is, in fact, comparable. (In addition, we have evidence that DNFB causes a 20% inhibition of the (Na+K)ATPase isolated from frog muscles, suggesting that some reduction of K influx could result from DNFB treatment, and that the actual discrepancy between Na influx 'gained' and K influx 'lost' may be even larger than observed here.) In any case, Na:K exchange is not much affected by elevation of ADP levels, despite marked stimulation of pump-mediated Na:Na exchange.

To summarise, our experiments were designed to determine whether Na:Na exchange occurs in addition to, or in substitution for, ongoing Na:K exchange. We examined whether ouabain sensitive membrane potential and strophanthidin-sensitive K influx (both indices of pump-mediated Na:K exchange) could be inhibited by induction of Na:Na exchange, and found little evidence of inhibition. Our conclusion is that Na:K exchange can continue unaltered when Na:Na exchange is induced by the presence of ADP. This effect of intracellular ADP on the mode of operation of the sodium pump is in marked contrast to that of external potassium. Garrahan and Glynn² have shown in red blood cells that reduction of $[\text{K}]_o$ stimulates Na:Na exchange at the expense of Na:K exchange, and we have similarly found that raising $[\text{K}]_o$ to 10 mM reduces pump-mediated sodium influx into DNFB-treated skeletal muscle to low levels¹⁷. Further strong evidence in favour of interconvertibility of Na:K and Na:Na exchange is provided by the work of Bodemann and Hoffman¹⁸.

The following qualitative kinetic picture of the sodium pump can now be drawn. In muscle cells with normal (that is, low) levels of ADP, bathed in K-free Ringer's solution, sodium

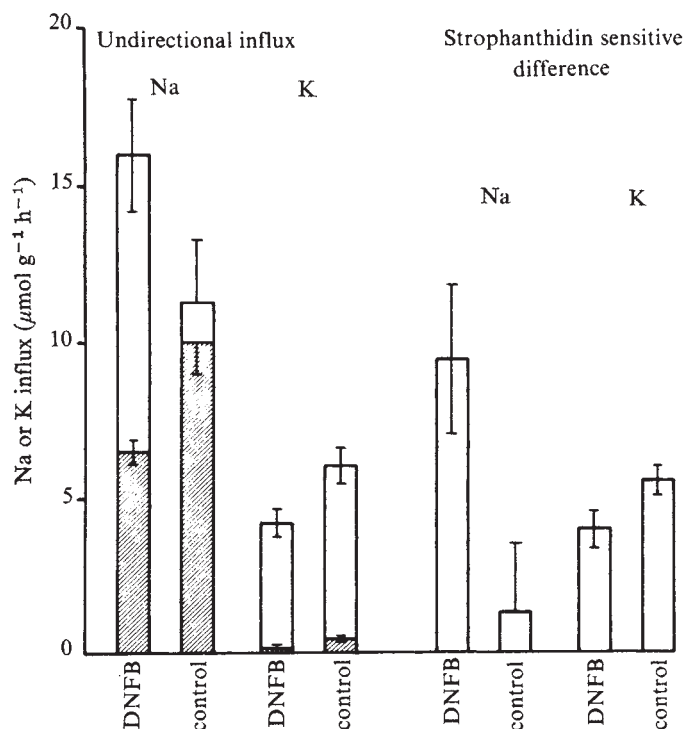


Fig. 2 Simultaneous determination of Na and K influxes. Values are means $\pm$ s.e.m. Protocol as for Fig. 1 except that both ^{22}Na and ^{42}K were present in the labelling solution, and that $[\text{K}]$ was kept at 2.5 mM. At this $[\text{K}]$, ouabain sensitive Na influx is reduced to about two thirds of its maximum value observed in K-free Ringer's. Hatched areas represent fluxes in the presence of 10^{-4} M strophanthidin. Washout samples were counted to determine total counts and then recounted after decay of the ^{42}K (more than 11 half lives). We found no long-lived contaminant in the ^{42}K used. Na influx values were not corrected for efflux during the uptake period, since the rate coefficient for efflux in DNFB is not constant⁹. Efflux is smaller in untreated muscles than in those treated with DNFB, however. Thus the strophanthidin-sensitive Na influx in DNFB will be underestimated (using the peak rate constant for efflux in DNFB, this underestimation was calculated to be about 20%) to a greater extent than that in untreated muscles. Consequently the stimulation of Na:Na exchange should, if anything, be even more pronounced than the uncorrected data suggest.

pump molecules are mostly inactive. As the external potassium concentration is raised, pump activity will increase toward saturation, thus reducing the silent fraction. At a given $[\text{K}]_o$, the silent fraction can be made to engage in Na:Na exchange to an extent determined by intracellular $[\text{ADP}]$, while the previously active fraction continues to engage in Na:K exchange. Further elevation of $[\text{K}]_o$ in the presence of high intracellular ADP levels continues to recruit an increasing fraction of the pump population into Na:K exchange, but now at the expense of both the silent (if any) and the Na:Na exchanging fraction. Thus, induction of Na:Na exchange by ADP is additive with respect to ongoing Na:K exchange, as shown here, but induction of Na:Na exchange by removal of external potassium² is substitutive with respect to ongoing Na:K exchange.

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Independent mechanisms of cyclic AMP and glucocorticoid action

IN eukaryotic cells, cyclic adenosine monophosphate (cyclic AMP) acts as the intracellular mediator for several hormones (for example, adrenaline and certain polypeptide hormones)¹ and plays an important part in the modulation of cell proliferation^{2,3}. This cyclic nucleotide is known to function by activating a protein kinase^{4,5} which mediates the phosphorylation of specific proteins, thereby modifying their biological activity. The activation of protein kinase may, in fact, be the sole mechanism of action of cyclic AMP in mammalian cells^{6,7}. Glucocorticoids, and steroid hormones in general, act through a mechanism^{8,9} which involves the binding of the hormone to specific cytoplasmic receptor proteins, the translocation of the receptor-steroid complex into the cell nucleus and the association with chromatin. The cellular responses to glucocorticoids and cyclic AMP are quite similar in several systems and the potentiating effect of the steroid on cyclic AMP-mediated processes is often referred to as 'permissive effect' (for reviews see refs 10 and 11). In several cell types glucocorticoids have been reported to increase, although moderately, the intracellular concentration of cyclic AMP (refs 12–15). These observations suggest that the cyclic nucleotide and glucocorticoids could have some distal step(s) in common in their mechanism of action. We have tested this hypothesis by cloning responsive cells in the presence of both effectors and report here that resistance to cyclic AMP and glucocorticoids occurs independently.

Our experiments were carried out with cultures of S49.1 mouse lymphoma cells in which both glucocorticoids and cyclic AMP (or its dibutyl derivative, db cyclic AMP) inhibit cell proliferation and, on prolonged exposure, cause the cells to die^{16–19}. Mutant cells resistant to growth-inhibitory and cytolytic effects have been isolated and most of them have defects related to known steps in the action mechanism of the two effector types^{7,20–23}. One would therefore expect that most of the mutants of each type

Fig. 1 Dexamethasone binding in cytosol preparations of S49.1 clones. The clones (experiment of Table 2) grown in the presence of db cyclic AMP and dexamethasone (Δ , ∇) as well as representative clones from the other groups of plates (no selection, $\bullet$; selected in db cyclic AMP, $\circ$; selected in dexamethasone, $\square$) were grown up in suspension cultures. Cytosols were prepared and incubated with various concentrations of ^3H -dexamethasone (26 Ci mmol⁻¹, Amersham)²⁷. The specifically bound steroid is plotted as a function of free steroid.

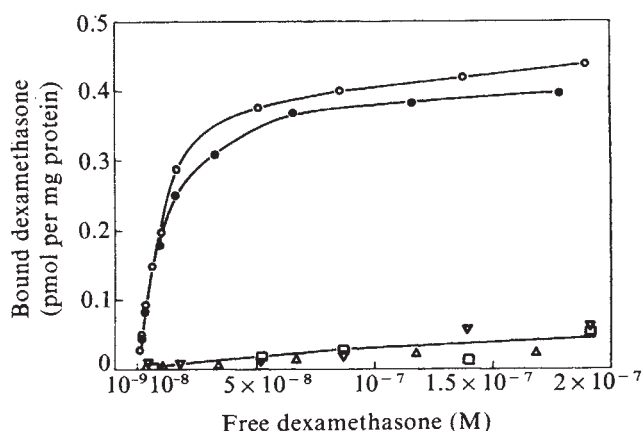


Table 1 Sensitivity of S49.1 clones isolated by single-step selection

Cloning in	Number of clones tested	Number of clones sensitive to Dexamethasone	Number of clones sensitive to db cyclic AMP
Dexamethasone	8	0	8
db cyclic AMP	20	20	0

S49.1 cells¹⁶ were grown in Dulbecco's modified Eagles' medium supplemented with 10% foetal calf or horse serum and cloned over a feeder layer of mouse embryo fibroblasts^{24,25} in soft agar containing 10^{-7} M dexamethasone or 0.5 mM db cyclic AMP and 0.2 mM theophylline. Clones were grown up and tested for sensitivity in suspension culture by seeding cells at a density of 3×10^5 ml⁻¹ in medium containing either 10^{-7} M dexamethasone or 0.5 mM db cyclic AMP and 0.2 mM theophylline. After 3 d of incubation, cell viability was determined with Trypan blue at a concentration of 0.2%. Clones were designated sensitive if > 90% of the cells stained blue. At least 90% of resistant cells or cells incubated without drug excluded the dye.

should not show cross resistance. This is seen in Table 1 for several S49.1 clones which have been isolated by independent procedures. Since most glucocorticoid-resistant mutants carry defects in the steroid receptor^{22,23}, the possibility that the steroid receptor is involved directly in the response to the cyclic nucleotide is excluded. There could exist at a relatively low frequency, however, a subclass of mutants which differ from those described thus far and are defective in some mechanism common to both glucocorticoids and cyclic AMP. Such cells should be doubly resistant and should be selected preferentially if both agents were used together.

Table 2 shows a cloning experiment in which both the steroid and db cyclic AMP were used. Here the cloning efficiency was extremely low compared with cloning in the presence of either one of the agents; only two colonies were seen. Significantly, this incidence is nearly the product of the frequencies at which singly resistant clones occurred.

The most plausible explanation for this observation is that the 'doubly resistant' cells contain two independent mutations. These cell clones were therefore examined for the phenotypes of resistance. The cells of both clones were identified as deficient in glucocorticoid-specific receptors (Fig. 1). In extracts of these cells however, protein phosphokinase activity was stimulated by cyclic AMP in a manner indistinguishable from the wild type (data not shown). Growth experiments in suspension cultures revealed that the two clones obtained in the double selection experiment of Table 2 were still growth inhibited by the cyclic nucleotide (Fig. 2). The half-maximum effect was between 0.05 and 0.1 mM db cyclic AMP (data not shown). Consistent with this growth inhibition, these clones appeared as very small colonies in the experiment of

Table 2 Incidence of resistance to dexamethasone and db cyclic AMP

Agent present during cloning	Cells applied per plate	Colonies grown per plate	Mean frequency of resistant cells*
Dexamethasone db cyclic AMP			
— —	10^8	45, 50, 53	
— —	10^4	22, 24, 27	5.0×10^{-3}
+ —	10^6	45, 49, 52	9.9×10^{-5}
— +	10^6	1, 1, 0, 0, 0	4.1×10^{-7}
+ +		0, 0, 0, 0, 0	

*Corrected for plating efficiency of 49%.

Recently cloned S49.1 cells were treated for 24 h with $0.75 \mu\text{g ml}^{-1}$ ICR 191 (a gift from Dr H. R. Creech). Surviving cells were allowed to grow up in the absence of drugs and were used for the cloning experiment (compare with Table 1). Plates with additions contained dexamethasone at 10^{-7} M and 0.5 mM db cyclic AMP plus 0.2 mM theophylline. The expected frequency of doubly resistant mutants is $5.0 \times 10^{-3} \times 9.9 \times 10^{-5} = 5.0 \times 10^{-7}$ if each marker is acquired in a distinct and independent step²⁶.

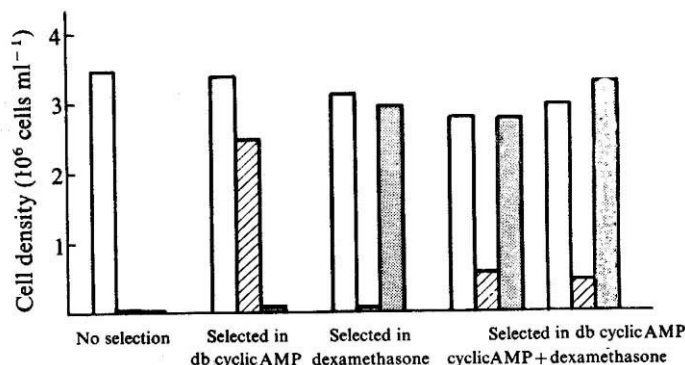


Fig. 2 Growth of S49.1 clones in the presence of db cyclic AMP and dexamethasone. Clones from the experiment of Table 2 (the same as used for Fig. 1) were grown in suspension culture and seeded at a cell density of 2×10^5 ml⁻¹ in medium containing either 10^{-6} M dexamethasone (stippled bars) or 0.5 mM db cyclic AMP and the phosphodiesterase inhibitor RO 20-1724 at a concentration of 30 μM (hatched bars). The density of viable cells was determined after 72 h. Control cultures are represented by open bars.

Table 2 and would probably not have been picked for further propagation in a single selection experiment in which a fair number of more highly resistant mutants with defects in protein kinase are routinely recovered²⁵. The preferential accumulation of cells in the G₁ phase of the cell cycle was observed by flow cytometry²⁸ on exposure of the mutant cells to the cyclic nucleotide (data not shown). While 0.3 mM db cyclic AMP completely arrested wild-type cells in G₁ after 24 h (ref. 17), the effect on the mutant cells was less pronounced. This is consistent with the intermediate growth inhibition (Fig. 2). These growth characteristics and the presence of cyclic AMP-responsive phosphokinase show phenotypic similarities between these mutant clones and the recently isolated cyclic AMP unresponsive 'deathless' cells²⁹. These 'deathless' mutants are growth inhibited, but not lysed, by cyclic AMP, however, they are fully sensitive to steroid-induced cytolysis.

The experiments described here indicate two independent lesions for steroid resistance and decreased cyclic AMP sensitivity, respectively, in the 'doubly resistant' mutants. This implies that there is no common mutable biochemical step in the two processes from effector-cell interaction to cytolysis in the cell system studied here. We cannot exclude, however, the possibility that mutations in a biochemical process common to cyclic AMP and steroid-mediated cytolysis occur at a frequency too low to be detected by our technique. Alternatively, such a common biochemical process could be required for cell viability. If so, mutations affecting that putative process would result in cell death and mutants could therefore not be obtained, except as conditionally lethal mutants. Nevertheless, there is synergism between cyclic AMP and glucocorticoids in S49.1 lymphoma cells. If these cells are pre-exposed to db cyclic AMP and thereby arrested in the G₁ phase of the cell cycle they are much more susceptible to the cytolytic effect of the steroid than untreated cells¹⁸. There must therefore exist, at some level, interaction between both effector systems in these cells as well as possibly in other mammalian cells.

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The shape of the ultraviolet inactivation curve for transforming DNA

THE very unusual inverse square kinetics of ultraviolet inactivation of transforming DNA were first reported by Rupert and Goodgal¹, and subsequently there was speculation that these kinetics result from blocks to recombination formed by ultraviolet lesions in the DNA² (ref. 2). We show here that when the recipient cell lacks both excision and postreplication repair, the ultraviolet inactivation of transforming DNA becomes a simple exponential rather than an inverse square relation. The data suggest that the square root kinetics observed with repair-proficient recipient cells result from repair processes themselves rather than from the recombination between irradiated transforming DNA and recipient DNA.

The inverse square relation, shown for transforming DNA from *Haemophilus influenzae*¹ and *Bacillus subtilis*², is: $\sqrt{(N_0/N)} = 1 + CD$, where N_0 and N are the number of transformants from unirradiated and irradiated DNA, respectively, D is the dose and C is a constant which depends on the genetic marker, the particular DNA preparation, the wavelength of the radiation and the repair capacities of the host^{1,3,4}. The equation fits the data except for very large doses, where the inactivation becomes close to exponential¹. Two theoretical formulations for the inactivation of transforming DNA have been based on the idea that recombination of a piece of DNA can take place up to but not including an ultraviolet lesion^{2,6}. After the first increment of dose, the 'targets' for further inactivation are the sections of DNA molecules between the genetic marker and the nearest lesion. Further inactivation takes place only when a lesion occurs in one of these sections. Thus with increasing dose the target decreases in size, and a larger and larger increment of dose is necessary for further inactivation. The theoretical formulations, although slightly different, both approximate to the empirically obtained equation, but are based on an incorrect assumption, namely that pyrimidine dimers in transforming DNA, responsible for almost all the inactivation by ultraviolet radiation^{5,7}, are not integrated into the recipient genome.

It was first shown that dimers are integrated into *H. influenzae* recipient DNA by a demonstration that irradiated transforming DNA which was integrated by the criterion of marker linkage could be enzymatically photoreactivated⁸. It was later found that integrated DNA containing dimers can kill the recipient cell⁹, and that excision of dimers takes place only after the damaged DNA has been integrated^{10–13}.

Excision repair might be considered to be responsible for the square root relationship, with the following rationale. Because transforming DNA is integrated into the recipient genome as a single strand¹⁴, there is a finite probability that excision repair not only may eliminate damage, but may also eliminate the genetic marker assayed in transformation. Following a given increment of dose, whether the repair process eliminates the marker or helps the marker to survive depends on the distance between the marker and the nearest ultraviolet lesion (usually a pyrimidine dimer), and also on the length of the region excised, assumed to be variable from one repaired region to another. After a second increment of dose, the only transforming DNA molecules with a still lower probability for transformation are those with an ultraviolet lesion between the marker and the previous lesion. But, excision repair alone cannot provide the explanation for the shape of the inactivation curve for transforming DNA, since the square root relationship also holds for recipients that are lacking in excision ability^{4,15,16}.

It has been suggested that postreplication repair might be the cause of some loss of donor genetic information, and thus be responsible for the shape of the inactivation

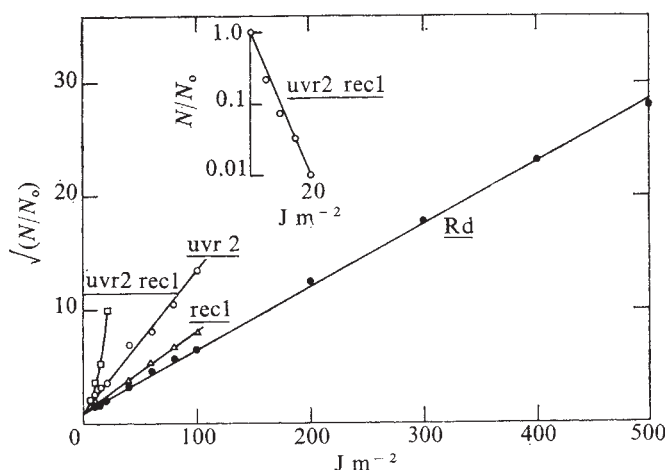


Fig. 1 Inactivation of transforming DNA assayed on four different streptomycin-sensitive *H. influenzae* strains, *Rd* (wild type), *recl-1* (ref. 20), *uvr2* and *uvr2recl* (ref. 21). N and N_0 are the number of transformants obtained from irradiated and unirradiated DNA. Transforming DNA from cells resistant to 250 $\mu\text{g ml}^{-1}$ streptomycin was purified as previously described²² and irradiated at 10 $\mu\text{g ml}^{-1}$ in 0.15 M NaCl plus 0.015 M trisodium citrate. Irradiation at 254 nm was performed as before⁴. Cells were made competent by a modification of Stuy's method²³. They were grown with aeration in supplemented brain heart infusion medium⁴ to an optical density of 0.44 at 675 nm, and then left standing at 37 °C for 90 min without aeration. They were then diluted a factor of eleven into 10 mM phosphate buffer, pH 7.0, plus 0.15 M NaCl, and shaken at 30 °C for 60 min. They were next exposed to 1 $\mu\text{g ml}^{-1}$ transforming DNA at 37 °C for 30 min. Growth medium (2 to 5 ml) was then added to each 1 ml of cells, and the cells were grown for 2–3 h before being plated in streptomycin-containing agar medium. The larger volume was used for the *recl* strains, in order to obtain statistically significant numbers of transformants, and ten platings of 1 ml each were made for each point. Each of these assays (all obtained with the same set of irradiated DNAs) were repeated at least three times and the data averaged. The minimum number of *recl* or *uvr2recl* colonies counted per point was 70. The approximate frequencies of transformation by unirradiated DNA in these experiments were 0.7×10^{-2} (*Rd*), 0.5×10^{-2} (*uvr2*), 0.5×10^{-7} (*recl-1*) and 0.2×10^{-7} (*uvr2recl*).

curve¹⁴. Postreplication repair in *H. influenzae* has been shown to involve extensive exchange between new and old strands¹⁷, during which the transforming DNA marker integrated into only one strand could be eliminated with a probability dependent on the distance between the marker and the nearest lesion, and on the (variable) length of the exchange. A test of the hypothesis is to measure the survival of irradiated transforming DNA on a mutant strain such as *recl* (ref. 18) deficient in postreplication repair^{17,19}. Earlier attempts at this experiment were defeated by the extremely poor transformation of the mutant (around 10⁻⁶ the frequency of the wild type). Recently it has been found that with a different competence regime, the transformability of the *recl* strains, but not other strains, is markedly improved²⁰, making the measurement feasible.

Figure 1 shows data plotted in the square root plot for ultraviolet inactivation of transforming DNA assayed on four different *H. influenzae* recipients. The data on three of the recipients, the wild-type (strain *Rd*), postreplication repair-deficient *recl* and excision-deficient *uvr2*, fit the straight line relationship, whereas on the double mutant *uvr2recl* recipient the data deviate from the straight line. Moreover, the transforming DNA assayed on *uvr2recl* seems to be inactivated exponentially, as seen in the insert of Fig. 1.

I interpret the data to mean that excision and/or postreplication repair in the recipient is responsible for the inverse square relationship. When repair is absent the inactivation becomes exponential, as though there is a critical region of DNA surrounding the marker which must be free of damage for transformation to be successful.

Excision repair increases survival of transforming DNA more than does postreplication repair, as seen in the fact that the DNA assayed on *recl* seems only a little more sensitive than on the wild-type strain *Rd*, but on *uvr2* the DNA seems considerably more sensitive. Postreplication repair in *H. influenzae* is not an inefficient process, as judged by its effect on survival following ultraviolet irradiation of cells²¹. Thus postreplication repair may eliminate the transforming DNA marker almost as often as it increases its survival.

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Psoralen and near ultraviolet light; a probe for study of control of protein synthesis

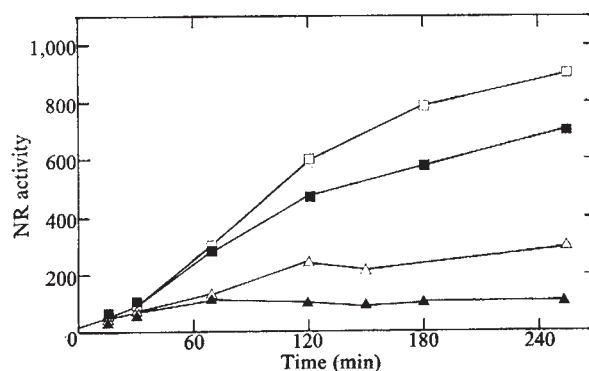
The study of the control of protein synthesis in eukariotic cells usually involves the use of chemical inhibitors of macromolecule synthesis¹. The drawback of these procedures is that the inhibitors have side effects which do not

allow conclusive interpretations. Furthermore, the precise timing of the inhibitor effect is not always possible due to the lack of knowledge of the effective *in vivo* concentration and the rate of its entry into the cells. Furocoumarins (psoralens) photosensitise biological systems to near ultraviolet (NUV) light (300-400 nm) (refs 2-4). Psoralen and its derivatives react with pyrimidine bases in DNA when irradiated with NUV light to form monoadducts and DNA crosslinks^{5,6}, the latter being the main lesion responsible for the biological damage in bacteria and mammalian cells^{4,7}. Since the photoreactions in the cells are specifically with DNA^{8,9} and lead to its immediate inactivation as a template for DNA and RNA synthesis⁸, we have considered the use of this system for the study of control of protein synthesis. The degree of inactivation of DNA can be easily controlled by varying the NUV light dose, thus avoiding the complications involved in the use of drugs.

The well documented¹⁰ development of nitrate reductase (NR) activity in the XD cell line of tobacco, in response to nitrate, was chosen as the system under study. The cells were grown as previously described¹¹ on chemically defined, nitrate-less M-ID medium supplemented with 5 mM L-glutamine (Gln cells) as a sole nitrogen source. Cells were grown to mid-logarithmic phase, collected by filtration and resuspended in fresh nitrate-less M-ID medium at 1 g per 10 ml. 4,5'-8-trimethylpsoralen (Paul B. Elder Co.) was added to a final concentration of 5×10^{-6} M. The cells were incubated in the presence of psoralen for 5 min, and then irradiated for the indicated duration with two tubular fluorescent black light lamps (UV Products, lamp 50058) held in a reflector. Maximum emission of the lamps was at about 360 nm and the incident flux was $20 \text{ J m}^{-2} \text{ s}^{-1}$ as measured by a calibrated black light meter (Model J-221, UV Products). During irradiation, the cell suspensions were continuously stirred. At the end of the irradiation period, samples of the cell suspensions were removed and were either diluted into nitrate-less M-ID medium supplemented with 10 mM KNO₃, or kept undiluted and given KNO₃ to a final concentration of 10 mM, when induced, or non-induced cells were used, respectively. Cells not treated were used as control. Following irradiation, the cells were shaken for the duration of the experiment. The NR activity *in situ* was determined at time intervals by the procedure described by Ferrari *et al.*¹². Enzyme activity is expressed as nmol NO₂⁻ formed per h per g fresh weight.

The effect of psoralen + NUV light on the induction of NR activity induced by nitrate in Gln cells is shown in Fig. 1. Increasing the radiation dose reduced the rate of develop-

Fig. 1 The inhibitory effect of psoralen plus NUV light on the induction of NR activity. Cells were grown in nitrate-less M-ID medium supplemented with 5 mM L-glutamine. The cells were collected and resuspended in nitrate-less M-ID. KNO₃ (10 mM) was added to part of the cell suspension and used as control (□). The rest of the cell suspension was exposed to psoralen (final concentration 5×10^{-6} M) and irradiated for 1 min (■); 3 min (△), and 5 min (▲). KNO₃ was added to the treated cells (final concentration 10 mM) at the end of the irradiation period. The NR activity *in situ* was determined at the indicated times.



ment of NR activity and markedly lowered the steady state level of the enzyme. The two effects could be observed as early as 15–30 min after exposure of the cells to nitrate. There was no effect of either psoralen alone or irradiation alone (data not shown). Only the combination of psoralen and NUV light at the proper dose was inhibitory to the development of enzyme activity.

The effect of psoralen plus NUV light on partially induced cells is shown in Fig. 2. Irradiation for 1 min strongly inhibited further development of the enzyme as compared with control cells. Higher radiation doses caused the initial level of activity to decline in time.

Based on the target specificity of psoralen plus NUV light⁸ we interpreted the results shown in Figs 1 and 2 as follows. Exposure of cells to psoralen plus NUV light just before nitrate addition (non-induced cells) or during the induction process (induced cells) inhibits the synthesis of functional mRNA, thereby inhibiting the development of NR activity. We further suggest that in cells growing on L-glutamine as a nitrogen source there is none or very little mRNA coding for the NR protein complex or for an essential subunit of the enzyme. If mRNA coding for the enzyme were present in Gln cells one would expect an initial development of NR activity upon exposure of the cell to nitrate, which is insensitive to the effect of psoralen plus NUV light. The inhibitory effect of psoralen plus NUV light would be observed gradually as old mRNA decays and the synthesis of new mRNA is inhibited. Such a situation is summarised in Fig. 3. It was previously shown¹³ that tungsten (W), an analog of molybdenum (Mo), inhibits the development of NR activity in XD cells due to the formation of non-functional enzyme. The non-functional enzyme can be made functional to a certain extent *in vivo* in the absence of protein synthesis (presence of cycloheximide) by adding excess Mo. Thus, it was assumed that the mRNA present in W-treated cells and translated to a non-functional enzyme, would be translated to a functional enzyme following exposure to psoralen plus NUV light and addition of Mo. It can be seen from Fig. 3 that this was indeed the case. The low level of NR activity in cells which were treated with W increased following exposure to psoralen + NUV light and addition of excess Mo. The increase cannot be accounted for by the activation of pre-existing non-functional enzyme, as can be judged by cells treated with cycloheximide before the addition of Mo.

We conclude that exposure to psoralen plus NUV light inhibits the synthesis of mRNA coding for NR or an essential subunit of the enzyme, thereby inhibiting the synthesis of the enzyme. The translation of pre-existing NR is not affected. We suggest that the combination of psoralen

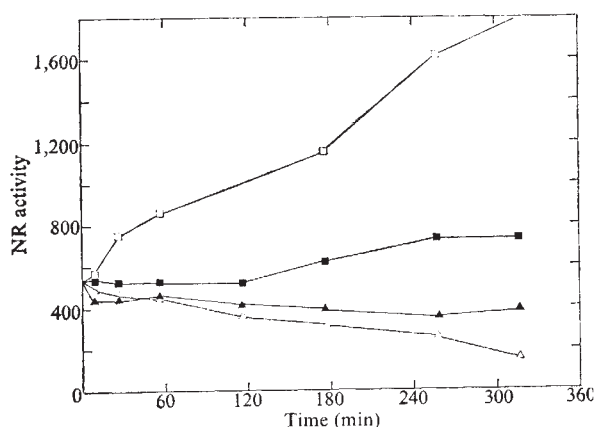


Fig. 2 The effect of psoralen plus NUV light on NR activity of partially induced cells. Cells were allowed to develop NR activity for 24 h and were then collected and treated as described in Fig. 1. Control (□), 1 min irradiation (■), 3 min irradiation (▲), 5 min irradiation (△).

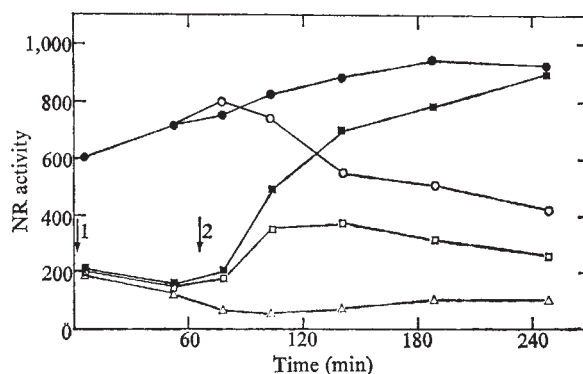


Fig. 3 Translation of pre-existing mRNA following exposure to psoralen plus NUV light. Cells were allowed to develop NR activity in the absence (●, ○) and presence (■, □, △) of 2×10^{-4} M sodium tungstate. At zero time, arrow no. 1, cycloheximide was added to one of the cell suspensions (△) to a final concentration of $10 \mu\text{g ml}^{-1}$. Arrow no. 2 indicates: first, exposure of the cells to psoralen plus 5 min irradiation (○, □), and second, the addition of sodium molybdate to a final concentration of 2×10^{-4} M to all of the cell suspensions (●, ○, ■, □, △). At the indicated times NR activity *in situ*, was determined.

and NUV light can be used to distinguish between transcriptional and post-transcriptional controls of protein synthesis.

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Genetic recombination through protoplast fusion in *Streptomyces*

MEMBERS of the genus *Streptomyces* yield over 60% of known antibiotics¹, including more than 70 commercial products², as well as at least one very important enzyme (glucose isomerase). Members of the related genera *Nocardia* and *Micromonospora* produce some further valuable antibiotics (rifamycins, gentamicins). Recombination through conjugation is widespread in these actinomycetes³ but only to a very limited extent has it been applied to strain improvement. The main reason for its neglect has probably been the necessity to introduce selectable (for example drug resistance) or more generally counter-selectable (auxotrophic) markers into the strains to be crossed in order to identify recombinants occurring at frequencies of 10^{-6} or lower among predominantly asexual progeny. The marking of strains is time-consuming; moreover it may induce deleterious mutations along with the markers, and these will usually go undetected since auxotrophic mutations themselves often depress antibiotic yield. In certain cases, recombination occurs so rarely that single markers in each parent cannot be used to distinguish it from reverse mutation⁴, while in some strains

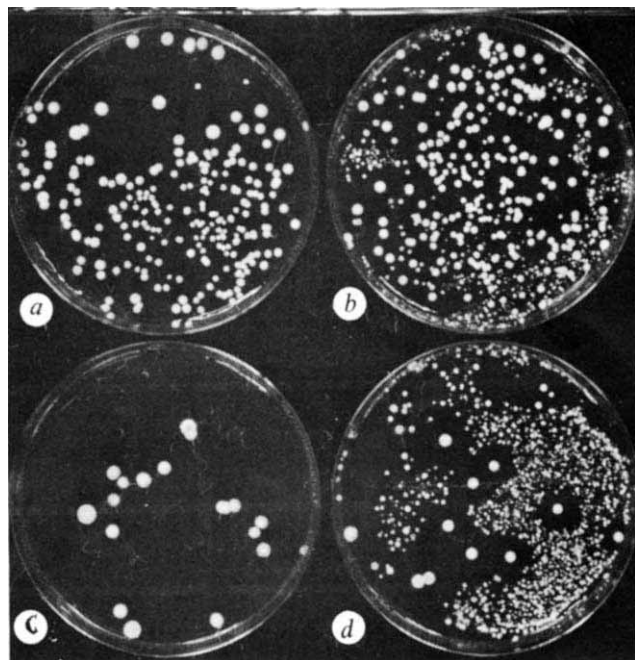


Fig. 1 Platings of *S. acrimycini* protoplasts on regeneration medium after dilution in water (a, c) or medium P (b, d) at dilutions 10^{-1} (a, b) and 10^{-2} (c, d). Note the large colonies derived from osmotically stable non-protoplasted units, occurring in equal numbers on both series of plates, and the much more numerous colonies arising from regenerated protoplasts after dilution in medium P. The latter are severely inhibited, especially at lower dilution, by the precociously developing non-protoplast colonies.

no recombination has been detected³. The availability of a simple generally applicable procedure to recombine actinomycete strains at high frequency would therefore greatly facilitate the routine use of recombination in strain improvement. We report here such a procedure for *Streptomyces* which depends on polyethylene glycol (PEG)-induced protoplast fusion and regeneration. Recombination frequencies achieved by this technique are so high that selectable markers could be dispensed with, at least in certain kinds of strain improvement programmes.

Protoplasts of five species were prepared and regenerated by procedures slightly modified from those of Okanishi, Suzuki and Umezawa³. 25 ml samples of medium S containing 1–4% glycine, depending on the strain, were inoculated with 10^7 – 10^8 spores and incubated with shaking in 250 ml Erlenmeyer flasks at 30 °C for 3–4 d. Growth was progressively retarded, and the efficiency of conversion of mycelium to protoplasts increased, with increasing glycine concentration. For routine protoplast preparation, mycelium was therefore collected from flasks with a glycine concentration which markedly inhibited growth but which allowed a reasonable yield of mycelium. After two washes at room temperature with 0.3 M sucrose, mycelial pellets (usually consisting of spherical aggregates about 1 mm in diameter) were re-suspended in 1.5 ml of lytic enzyme mixture and incubated at 32 °C until clouds of protoplasts were liberated from the mycelial mass on gentle swirling of the liquid (60–90 min). 4 ml of medium P was added; the suspension was pipetted vigorously several times to aid release of protoplasts from the still largely coherent mycelial mass, and filtered through cotton wool (sometimes followed by Nucleopore filtration). The protoplasts were pelleted by centrifugation at 1,000g for 5–10 min, resuspended in 4 ml of medium P and again pelleted and resuspended.

For studies of protoplasting and regeneration efficiency,

samples of the suspensions were diluted in parallel in medium P (which contains 0.3 M sucrose) or in water and plated on R2 regeneration medium supplemented with required growth factors. (In preliminary work, the R1 and R2 media³ were assessed; somewhat higher regeneration frequencies were obtained with R2 and this was therefore adopted.) Colonies from water-diluted suspensions arose from osmotically stable and presumably non-protoplasted units (mycelial fragments produced by sonic disruption of control cultures equilibrated in medium P were 100% osmotically stable), while the excess of colonies from comparable dilutions in medium P represented regenerated protoplasts. For some strains, non-protoplasted units developed precociously so that colony counts after dilution in water or medium P were at first the same; later, numerous protoplast colonies appeared on the plates that received osmotically protected suspensions. In most strains the protoplast colonies soon reached the size of the non-protoplast colonies. In *S. acrimycini*, however, non-protoplast colonies inhibited development of the protoplast colonies, which were largely suppressed on high-density plates (Fig. 1). Non-protoplasted units typically represented 0.1–1% of total colony-forming units when the parent culture was grown with an optimal glycine concentration. With lower concentrations, the proportion was higher; it was essentially unchanged by filtration through 2.1- μ m Nucleopore filters after the routine cotton-wool filtration and so this step was usually omitted. Protoplasts varied so greatly in size, many possibly being too small to contain a genome, that little confidence was placed in microscopic counts; rough estimates of regeneration frequency were lower than the maximum of 40–50% reported by Okanishi *et al.*³.

Protoplast fusion induced with PEG has been described in bacilli^{6,7} but we closely modelled our procedure on that of Pontecorvo, Riddle and Hales⁸ for animal cells. Equal volumes (0.5–2 ml: half to a fifth of the yield of a flask) of protoplast suspensions of the two strains were mixed and, after centrifugation, the pellet was thoroughly drained and rapidly suspended in 0.5 ml of PEG 1540 1w+1.4 volumes medium P containing 15% dimethylsulphoxide (DMSO). After 1 min, 0.5 ml of PEG 1w+3 volumes of medium P was added and, after a further 3 min, 4 ml of medium P. Control pellets were suspended in 5 ml of medium P. The suspensions (containing flocculent protoplast aggregates after PEG treatment) were centrifuged, each pellet was resuspended in 0.25 ml of medium P, and the liquid was poured over a single plate of R2 medium supplemented with all growth factors required by both parental strains. Gentle spreading of the suspensions was done with a loop, rather than a glass spreader which might have tended to disrupt protoplast aggregates. After incubation of the plates for 4–6 d, spores were collected from the confluent cultures and assayed for parental and recombinant genotypes by standard procedures⁹.

For four species, *S. coelicolor*, *S. lividans*, *S. parvulus* and *S. griseus*, recombinants recovered on a medium selecting markers at two loci represented 1–27% of the frequency of the minority parental class, and 1–6% of the sum of both parentals (Table 1). In *S. coelicolor* A3(2), the same high recombinant frequency occurred in the absence of both known sex factors, SCP1 and SCP2 (ref. 10), as in the presence of one (Table 1) or both, suggesting that the recombination occurring following protoplast fusion is independent of sex factor activity and should therefore occur even in wild types that lack sex factors. In *S. acrimycini*, the recombination frequency was only 3×10^{-4} , but this is readily explicable by the inhibition of protoplast by non-protoplast colonies (see above; Fig. 1); a minority of the latter contributed disproportionately to the total spore progeny on the regeneration plates. There was little or no evidence for significant spontaneous protoplast fusion

Table 1 Recombination in five streptomycetes

Strains*	Treatment	per minority parent	Recombinants per majority parent	per total
<i>S. coelicolor</i> A3(2) (SCP1 ⁻ SCP2 ⁺ × SCP1 ⁻ SCP2 ⁺)	Mating	1.0×10^{-4}	1.8×10^{-6}	1.7×10^{-6}
	Protoplasts (no PEG)	7.6×10^{-6}	7.4×10^{-6}	3.8×10^{-6}
	(PEG)	9.9×10^{-2}	9.1×10^{-2}	4.7×10^{-2}
<i>S. coelicolor</i> A3(2) (SCP1 ⁻ SCP2 ⁻ × SCP1 ⁻ SCP2 ⁻)	Mating	9.4×10^{-7}	2.0×10^{-7}	1.7×10^{-7}
	Protoplasts (no PEG)	3.2×10^{-6}	1.5×10^{-6}	1.0×10^{-6}
	(PEG)	20×10^{-2}	8.9×10^{-2}	6.2×10^{-2}
<i>S. parvulus</i> ATCC 12434	Mating	1.8×10^{-5}	1.0×10^{-5}	6.3×10^{-6}
	Protoplasts (no PEG)	3.0×10^{-6}	2.3×10^{-6}	1.3×10^{-6}
	(PEG)	1.2×10^{-2}	1.1×10^{-2}	0.6×10^{-2}
<i>S. lividans</i> 66	Mating	$< 1 \times 10^{-4}$	$< 1 \times 10^{-6}$	$< 1 \times 10^{-6}$
	Protoplasts (no PEG)	$< 1 \times 10^{-4}$	$< 4 \times 10^{-7}$	$< 4 \times 10^{-7}$
	(PEG)	27×10^{-2}	7.2×10^{-2}	6.0×10^{-2}
<i>S. griseus</i> CUB 94	Mating	1.0×10^{-4}	2.4×10^{-6}	2.3×10^{-6}
	Protoplasts (no PEG)	1.5×10^{-4}	2.1×10^{-5}	1.9×10^{-5}
	(PEG)	9.8×10^{-2}	1.1×10^{-2}	1.0×10^{-2}
<i>S. acrimycini</i> IPV 1610	Mating	9.0×10^{-5}	5.1×10^{-5}	3.2×10^{-5}
	Protoplasts (no PEG)	4.0×10^{-5}	1.7×10^{-5}	1.2×10^{-5}
	(PEG)	5.3×10^{-4}	4.9×10^{-4}	2.6×10^{-4}

Compositions (quantities per litre of final medium) of medium S (for mycelial growth), medium P (for protoplast formation) and R2 (for regeneration) were as follows⁵. Medium S: glucose, 10 g; Difco Bacto peptone, 4 g; Difco yeast extract, 4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KH_2PO_4 , 2 g; K_2HPO_4 , 4 g. Glycine was added to medium S at 1% for *S. coelicolor* and *S. lividans*, 1.5% for *S. parvulus*, 2.5% for *S. acrimycini* and 3.5% for *S. griseus*. Medium P: sucrose, 103 g; K_2SO_4 , 0.25 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.03 g; trace element solution (per litre: ZnCl_2 , 40 mg; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 200 mg; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10 mg; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 10 mg; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 10 mg), 2 ml; $\dagger\text{KH}_2\text{PO}_4$ (0.5%), 10 ml; $\dagger\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (3.68%), 100 ml; $\dagger\text{TES}$ buffer (0.25 M, pH 7.2), 100 ml. Medium R2: sucrose, 103 g; K_2SO_4 , 0.25 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10.12 g; trace element solution, 2 ml; glucose, 10 g; Difco casaminoacids, 0.1 g; Difco Bacto agar, 22 g; $\dagger\text{KH}_2\text{PO}_4$ (0.5%), 10 ml; $\dagger\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (3.68%), 80 ml; $\dagger\text{L-proline}$ (20%) 15 ml; $\dagger\text{TES}$ buffer (0.25 M, pH 7.2), 100 ml.

*CUB, collection of the University of Bradford; IPV, collection of the Istituto Patologia Vegetale, Milan; for *S. lividans* 66 see ref. 14.

$\dagger$ Added after autoclaving.

since recombination frequencies were similar in mixtures of protoplasts untreated with PEG and in standard crosses ('matings') made on supplemented R2 medium (Table 1); mating after regeneration could therefore have accounted for the results. A possible exception was *S. coelicolor* A3(2) in SCP1⁻SCP2⁻ condition in which recombination in matings occurs at very low frequencies¹⁰.

In studies of mammalian cells^{11,12}, DMSO has been found to shift the optimal PEG concentration for fusion from 50% to 40%, allowing the use of a less viscous reagent; this advantage, important for high molecular weight PEG, may be marginal with low molecular weight PEG (such as the 1,540 tested here). With SCP1⁻SCP2⁻ mixtures of *S. coelicolor* A3(2), we tested the effect of omitting DMSO, with or without an increase in PEG concentration from the standard 40% to 50%. At 40% PEG, DMSO caused a significant increase in recombination frequency, but this was largely compensated by raising the PEG concentration to 50%. These findings were strikingly comparable with those for mammalian cells. Since the standard procedure was consistently as good as or better than the variations on it, we saw no reason to depart from it.

Analysis of the segregation of non-selected markers amongst recombinants from six-factor crosses of *S. coelicolor* A3(2) derivatives (SCP1⁻SCP2⁺ × SCP1⁻SCP2⁺ or SCP1⁻SCP2⁻ × SCP1⁻SCP2⁻) was done by selecting recombinants inheriting markers at two loci on opposite sides of the circular linkage map with two non-selected markers in each half of the linkage map to divide each half into three approximately equal map intervals. In a mating, multiple crossover classes represented about 2% of total progeny, but after protoplast fusion they represented over 20%. This loosening of linkage (increased recombination per unit of map length) might in part reflect a more complete diploidy in the units produced by protoplast fusion than in the merozygotes arising by mating; another possible explanation is multiple rounds of recombination in the large units arising by multiple protoplast fusion and regeneration (an enormous increase in protoplast size was described⁹ before regeneration).

The technique described here should be useful in at

least some recombinational approaches to industrial strain improvement. Particularly important are the very high recombination frequencies achieved, even in the absence of detectable sex factor activity: a frequency of 6% recombinants inheriting one of two alleles at each of two loci is equivalent to a total 'sexual' progeny of 24%. Moreover, the observed linkage relaxation would lead to a much more nearly random assortment of genotypes in a cross than would normally be the case. Both features would be ideal in the crossing of divergent lines obtained by successive mutant selection in order to generate an assortment of new genotypes, including those in which positive gene interactions gave rise to superior performance, or in which groups of negatively interacting genes had been disrupted¹³. Once recombinant yields had been optimised for a given set of strains, by minimising contamination of protoplasts with non-protoplasted units and by equalising the frequencies of the two parental genotypes, the total non-selected progeny of protoplast fusion could be screened for desirable phenotypes without the need for selectable markers. When two parents differ by several mutations, the great majority of 'sexual' progeny are recombinant in respect of these mutations. Even with a comparatively modest total frequency of such progeny of 10%, the percentage of unimproved genotypes in the protoplast fusion procedure could be less than that of unimproved and disadvantageous genotypes which survive a typical mutational step in strain improvement.

Other potential contributions of the procedure to *Streptomyces* genetics would be in the synthesis of genotypes carrying combinations of known markers. A limiting step in the development of genetics in a new strain is the production of multiply-marked stocks by the successive induction of mutations, usually auxotrophs. In matings, two auxotrophic markers are lost by counter-selection whenever two lines are combined and at least three different markers are needed in each parent before recombinants can be selected carrying more markers than either parent. By protoplast fusion, the markers of two parents can be combined without loss, allowing the rapid construction of complex genotypes. Whether or not the technique described here can be applied to the other important genera of

actinomycetes, and to interspecific combinations, remains to be seen.

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Test of the ballistic hypothesis of movement and the 'point of no return'

How long does it take to stop or modify a movement once it has begun? Where there is an error in producing an action as intended (usually because of control-display incompatibility) subjects may correct it in less than their normal reaction time (RT) by proprioception¹ or efference-copy monitoring^{2–4}. Where subjects work from a visual (external) error signal, however, they are usually said to take a full RT to stop⁵ or correct^{6,7} ongoing movements, so that there is a 'point of no return', after which they are committed for about 300 ms to any action decided on⁸. During this time the action is ballistic at the perceptual-motor level^{6,9,10}. But in a recent tracking experiment¹¹ where subjects wrongly anticipated the target motion, and so began a movement in the wrong direction, the correction time to stop the action (CorT) was often much shorter than the normal RT to initiate movements, down to as little as 120 ms in some cases. This suggests that fast error correction can occur from a visual signal alone, at least to stop movements (as in an emergency). Repeating the experiment we find that movements can indeed be arrested soon after they begin, but only if the beginning is delayed; it always takes one RT from the occurrence of the error signal. We therefore infer that there is a 'point of no return' at the decision level, but that movements are not necessarily ballistic at the motor level.

The experiment requires subjects to track predictively a target moving in a regular sequence of steps. They are told to try to synchronise their movements with those of the target, and thus to begin their actions at about the time they anticipate it will move. The target is then occasionally made to jump in the direction opposite to that expected, and the time taken to stop wrongly-predicted movements at these 'surprise jumps' (SJs) measured. As control and display are compatible, the only error signal is visual, and CorTs for incorrect movements are directly comparable with both normal RTs and RTs for correct movements at SJs (where subjects do not make errors).

Fifteen students were tested using an oscilloscope display and joystick control (described elsewhere¹¹). The 3-mm target

Table 1 Reaction times for correct movements

Class of movement	Trials	N	Mean (ms)	s.d. (ms)
A Follow irregular pattern	1 and 7	270	211	44
B Follow regular pattern	2 and 6	270	173	70
C Predict regular pattern	3, 4, 5	360	–199	319
D Correct moves to SJs (median for each S)	4, 5, 6	15	214	36

circle jumped horizontally from the centre of the screen to points 30 mm to left or right, and back, pausing for about 2 s at outward points and 1 s in the centre. There were seven trials of 20 cycles (120 s) each. On trials 1 and 7 the pattern of jumps outwards from the centre was irregular, and subjects followed the target as quickly as possible. On all other trials the target jumped alternately left and right (except where SJs occurred in trials 4, 5 and 6). On trials 2 and 6 subjects followed the target; on trials 3, 4 and 5 they tried to synchronise their movement with it. RTs and CorTs (for outward movements only) were measured from ultraviolet records to an accuracy of 10 ms.

Table 1 lists overall RTs for correct movements in the various conditions. Each estimate is derived from six or eight movements made by each subject in one or two samples taken from the trials listed, except 1D where numbers varied between subjects and the median score for each subject was used for the calculation. (1D represents RTs for correct movements at SJs.) Within all these classes of movement initial ANOVAs showed that there were no significant differences due to samples, trials or directions of movement. RTs for conditions 1A and 1B are similar to those found elsewhere^{1,10,12}. 1C shows that subjects were predicting on trials 3, 4 and 5, since their RTs were mostly negative, and rarely longer than +120 ms.

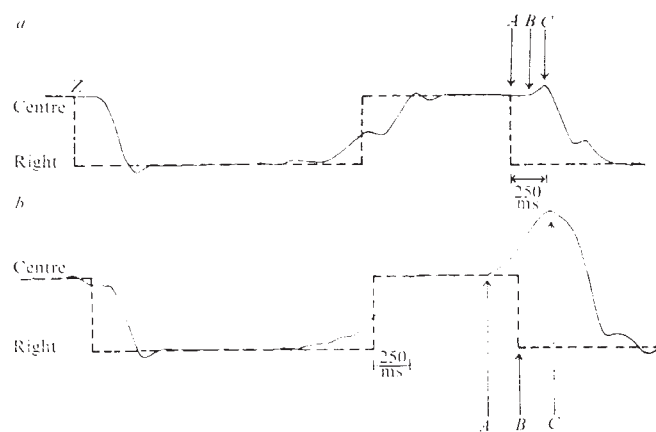


Fig. 1 Record tracing of wrongly-predicted movement. Dotted line, target; continuous line, subject. The track runs from left to right. *a*, At point Z the subject makes a normal aimed movement to the right, with an RT somewhat shorter than those to irregular target movements. At B he begins a movement to the left (expected direction of target jump) when the target in fact moves to the right again (point A). $A-B = RT$ (140 ms); $B-C = CorT$ (110 ms); this is much shorter than the average RT to follow the target, so the motor pattern initiated by an order from the voluntary decision mechanism is not completely ballistic once it has begun. It may be shut off during its execution if necessary, but only if it begins some time after the target jumps; the total time $RT + CorT$ is still at least one normal RT. *b*, At A the subject commits himself to a movement in what turns out at B to be the wrong direction. $A-B =$ negative 'RT' (–170 ms); $B-C = CorT$ (230 ms); this is about the same as the subject's normal RT. The $CorT$ $B-C$ here is about equal to the combined RT and $CorT$ of corrections in *a*. When the subject is committed to his action in advance of the error signal, therefore, $CorTs$ are not shorter than the normal RT, and are often rather longer.

1D shows that responses which correctly follow an unexpected direction of movement only do so after a lag of duration similar to the normal RT for irregular jumps 1A, that is, when the signal tells the subject which way to go. These RTs are significantly longer than those of 1B where the signal tells the subject when to go, the direction already being known ($t = 2.322$, $P < 0.05$ for 15 subjects).

Table 2 lists RTs and CorTs for incorrect predictive movements at SJs. Those begun after the target jumps (Fig. 1a) show evidence of relatively fast error correction (180 ms), this being roughly equivalent to RTs for the regular pattern (Table 1B), suggesting that a 'stop' order may take only as long as a simple 'go' order. The shortest CorT found in these records was 70 ms, and a dozen or so were less than 100 ms, which is shorter even than the time suggested for a motor system servo to adjust by some internal feedback¹³, so there may not be a minimum value of CorT for cutting off a movement in progress. Shorter CorTs than this, however, may be difficult to distinguish from natural tremor, although there was little evidence of the latter in these records—movements started and stopped quite distinctly on most occasions (for example, Z in Fig. 1a). Very short CorTs only occur, however, where the onset of the action is delayed, and the error signal therefore has time to begin its

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Table 2 Reaction times and correction times for errors at SJs

Class of RT/CorT	Trials	N	Mean (ms)	s.d. (ms)
A, Incorrect movements with positive RTs: (median for each S)				
—RT	4, 5, and 6	15	143	41
—CorT	4, 5, and 6	15	181	53
—RT+CorT	4, 5, and 6	15	309	52
B, Incorrect movements with negative RTs: (median for each S)				
—RT	4 and 5	15	–231	117
—CorT	4 and 5	15	309	98

passage through the central nervous system before the movement starts. When RT and CorT are added together (2A, bottom line), correction times again average about 300 ms from the time of the error signal.

Where incorrect movements begin in advance of the target jumping (Fig. 1b) subjects need about 310 ± 100 ms to stop an ongoing movement. This is close to the minimum value reported by others for stopping an action^{6–7}, or adjusting it through vision¹⁴.

These results suggest: (1) fast error-correction can occur from an external (visual) error signal alone (that is when an efference-copy explanation cannot apply); thus (2) movements are not necessarily ballistic in that they may be stopped, if found to be wrong, before they have run their full course. This takes about 180 ms on average, but sometimes only 70 ms or less. It occurs where subjects insert a delay between decision and action which partly bridges the normal RT; but (3) movements cannot be stopped in less than 300 ms from the occurrence of the error signal, that is, visual error correction does conform to the ballistic hypothesis at the highest perceptual-motor 'decision' level; (4) a predictive strategy reduces RT and gives better control where events are redundant, but lays the operator open to predictive error, the duration of which varies inversely with its initial RT. It must last a full RT, if, as in many skills, the action initiated from the prediction runs in time with the events predicted—this is the 'point of no return' of the wartime pilot, batsman or driver in an accident. Subjects working predictively, therefore, trade off a minimum RT for predictable events against the risk of a relatively long CorT for unexpected events when their predictions fail; the payoff values of errors and reactions will presumably determine their strategy in individual cases¹.

Lateralised perception of bilateral chimaeric faces by normal subjects

A COMPOSITE 'chimaeric' figure is one constructed by abutting two different half-figures along a vertical join. Following surgical section of the pathways connecting the two cerebral hemispheres (the commissures) patients subjectively experience a compelling perceptual 'completion' in their perception of both halves of such figures when presented at the midline of vision. This perceptual completion evidently occurs independently in each of the two cerebral hemispheres¹. The critical condition for this phenomenon might be the disconnection between the primary visual cortices of the two hemispheres. Only those portions of these cortical areas are cross-connected which represent a narrow strip of the visual field in the region of the vertical midline². We, therefore, tested normal subjects with chimaeric faces in which no vertical join was visible. Our subjects then experienced perceptual completion, as we had predicted.

There are at least three primary areas in the primate cortex which seem to be involved in the preliminary processes of visual pattern analysis, and which are known to be organised retinotopically^{3,4}. These three areas, known as V1, V2 and V3, give rise to commissural fibres which pass through the posterior splenium to corresponding areas in the opposite hemisphere. However, the cross-connected points are limited to the portions in each area which represent a strip of the visual half-field some 2–3° wide bordering the vertical meridian^{2–5}. One function which some of these fibres could perform is to assist the brain in its alignment of information deriving from the two visual half-fields⁶:

Table 1 Numbers of choices based on each constituent half-face (join visible)*

Response based on	Pointing (right hand)		Response condition Pointing (left hand)		Verbal	
	LHF†	RHF	LHF	RHF	LHF	RHF
Mean	6.92	6.50	9.67	7.00	6.42	5.83
S.D.	1.68	2.88	1.37	2.59	1.00	1.40

* The tabulated scores are based upon all 12 subjects of group A.

† LHF/RHF = left/right half-face.

Table 2 Numbers of choices based on each constituent half-face (joint hidden)*

Response based on	Pointing (right hand)			Response condition Pointing (left hand)			Verbal		
	LHF	RHF	Neither	LHF	RHF	Neither	LHF	RHF	Neither
Mean	4.50	5.00	2.50	7.00	3.13	1.88	2.57	3.43	1.00
S.D.	1.31	1.31	—	1.51	0.83	—	1.51	1.40	—

* The tabulated scores refer only to those subjects of group B who did not spot the stimulus asymmetry during the relevant test condition. There were eight such subjects in the manual response condition, and seven in the verbal response condition.

this might proceed by a constant process of subtractive cross-matching between similar feature-detecting units with correspondingly-placed receptive fields in the two adjacent half-field strips. The use of some such process could account for observations that an immediate impression of symmetry or asymmetry in a dot-pattern is based, other things being equal, on the symmetry or asymmetry of only the middle region of the array⁷.

If this argument is valid, then it might be these splenial fibres which enable the normal observer to detect readily the true nature of a centrally-flashed chimaeric figure. If so, then this detection should become very difficult if the central portion of such a figure were concealed; and since then no mis-match would occur, perceptual completion should be experienced.

We used two groups of 12 subjects, undergraduate students aged between 17 and 25. Both groups were tested comparably, viewing a series of chimaeric faces (made from colour photographs), each back-projected for 100 ms on a translucent screen at luminance levels of 30–40 Ft.L. A total of 24 chimaeric faces mixed with 12 normal faces were given first, the subject in each case being required to identify by pointing the face (or faces) he had seen, from amongst a total set of 12 possible faces. For the first half of these trials, the right hand was used in pointing, and for the second half the left hand. Next, a subset of six faces were given names, which the subject memorised. A second series of colour slides was then presented (seven chimaeric, six normal) with the subject in each case naming the face(s) that he had seen. The left/right occurrence of half-faces was balanced either within subjects (pointing condition) or between subjects (verbal condition). With one group of subjects (group A) conventional chimaeric faces were used, whilst with the other (group B), the same photographic originals had been overlaid with a vertical white strip of paper before the final photographic stage. The mean angular size of the faces (ear-to-ear) was 25°, and of the blank strip 5.3°. In all cases, the projected picture was centred on a spot on the screen which the subject was required to fixate.

The subjects of group A soon realised that they were viewing asymmetrical faces (between two and six chimaeric slides being required). Choices were not clearly biased in favour of the left or right half-face, except in the pointing condition where the left hand was used (Table 1). Analysis of variance of the pointing responses reveals a highly significant main effect of hand used ($F_{1,11} = 26.69$, $P < 0.001$), but this is confounded with a possible order effect. However, the significant interaction of hands $\times$ half-fields ($F_{1,11} = 9.00$, $P < 0.05$) reflects the fact that this improvement with change of hand was restricted to an increase in correct selections based on the left half of vision.

In group B, seven subjects never noticed that asymmetrical faces were being used, and the other five required between 11 and 29 chimaeric stimuli before recognising any asymmetry. Except beyond such a point, all subjects in group B indicated (manually or verbally) only one face on each trial. Subsequent careful questioning confirmed that they had always subjectively perceived a single complete face. There was a tendency for these 'completing' subjects

to name the face which had been exposed to the right half-field rather than that to the left, but this bias was not statistically significant (Table 2). However, analysis of variance on the choices made by pointing, showed a significant main effect of half-fields ($F_{1,7} = 7.43$, $P < 0.05$), in favour of the left. In addition there was also a highly significant interaction of hands $\times$ half-fields ($F_{1,7} = 38.45$, $P < 0.001$), again reflecting the fact that an asymmetrical distribution of choices appeared only when the left hand was being used for pointing.

Preventing normal subjects from seeing the join in chimaeric faces did not make them behave exactly like commissurotomy subjects. Nonetheless, perceptual completion did predominate in this condition, and several subjects even denied having seen any blank strip. Furthermore, completion evidently occurred irrespective of whether the half-face that determined response was in the left or right half-field.

We have also found that a girl with callosal agenesis, who is impaired in midline stereopsis⁸ (see ref. 9), showed perfect perceptual completion using conventional chimaeric figures (with the two halves directly abutting)⁹. This observation agrees with our present suggestions, if it can be argued that her brain has compensated, perhaps through the anterior commissure¹¹, for all of those visual cross-connections normally passing through the corpus callosum, except for those of visual areas V1, V2 and V3.

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matters arising

Dysmyelination in Jimpy mouse due to astroglial hyperplasia?

SKOFF¹ recently suggested a new explanation for the central dysmyelination in Jimpy mice. He suggested that astrocytic processes branch abnormally in these mutants before oligodendroglial proliferation. This branching continues until it surrounds most axons. It is further suggested that by this mechanism oligodendrocytes are prevented from reaching the axons, thus leading to secondary inhibition of oligodendroglial development and myelin formation. This concept, however, seems to be inadequately supported by the quantitative and qualitative data given.

The difference in the ratio of astrocyte cytoplasm to optic nerve tissue between hemizygous controls and Jimpys which were calculated in the planimetric study (ref. 1, Table 1) seems to be much too small to support the proposed thesis. A comparison against animals of a different strain is not valid. Considering the absolute cell counts (ref. 1, Table 2) the calculated surface area of six missing, non astrological cells (72 in hemizygotes, 66 in Jimpys) must be taken into account, if the ratio is to be interpreted as an expression of true astrocytic hyperplasia.

No illustration from the premyelination period showing the astroglial processes surrounding the axons is presented. The vast majority of axons in Fig. 2 ref. 1 are not in contact with astrocytic processes. Figure 3 in ref. 1 is irrelevant for Skoff's argument because it is obtained from optic nerve

at day 23, that is, 15 d after onset of myelination. The cited illustrations (ref. 2, Figs 7 and 8) are also irrelevant for the same reason. The effects which Skoff claims are probably concerned with pathologies following the primary event. The concept that reactive astrocytosis follows central nervous system pathology is well established.

Can astrocytic processes block oligodendrocytes from contacting axons? This hypothesis seems questionable. Blakemore²⁻⁵ produced degeneration of oligodendrocytes, demyelination and heavy astrocytosis in young mice with cuprizone. Demyelinated axons were ensheathed by astrocytic processes. Drug withdrawal led to clear remyelination induced by newly developing oligodendrocytes.

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SKOFF REPLIES.—Meier and Bischoff¹ say that the data used to support the hypothesis² that astroglial cells may interfere with myelination is inadequate. They claim that the astrocytic processes in Jimpy optic nerve do not contact the majority of the axons before the onset of myelination and that to show this condition afterwards is irrelevant. In the photograph of an 8-d-old Jimpy mouse optic nerve (Fig. 1 ref. 1), Meier and Bischoff state that large groups of axons are not contacted by astrocytic processes. Careful examination of this picture, however, shows that the large astroglial processes send out very fine branches into the fascicles of axons. I have calculated from the original photograph that 77% of the axons are contacted by astrocytic cytoplasm. At this stage, about 2% of the axons in littermates are in the process of being myelinated³. In my own 8-d material from Jimpy optic nerve, I have observed that the astrocytic processes not only contact the majority of the axons but they, in part, surround individual axons; this process continues throughout myelinogenesis². I have demonstrated that the abnormal branching of astrocytic processes in the optic nerve begins 2-3 d before the onset of myelin

formation. Since this astrogliosis (increase in processes and filaments) begins before myelinogenesis, it is reasonable to conclude that the astrocytic abnormality is not a reaction to abnormal myelination.

In response to the comments¹ that the ratio of astrocytic cytoplasm is too small to support the astroglial hypothesis, these ratios represent a 39% increase over the Swiss strain and an 18% increase over the heterozygote (the term hemizygote was used in the original paper). Concern over the effect of the missing cells on the above ratios is irrelevant since the area of the astrocytic cytoplasm was directly determined rather than by multiplying the number of astrocytes by an average area. The ratios were included to show that the total amount of astrocytic cytoplasm seems to be increased in electron microscopic preparations. It should be stressed that an increase in glial cytoplasm is not essential for an increase in the branching of their processes (two cells of a given volume can take on entirely different geometric configurations).

The Jimpy mice in this study were always compared with their littermates². The Swiss mouse was used as a control for the entire strain because gliogenesis seems somewhat abnormal in most animals of the litter. Electron microscopic observations of Jimpy littermates show varying degrees of astrogliosis in both optic nerve and spinal cord; the number of axons being myelinated in the branchial spinal cord also varies considerably in littermates of identical body weights.

The model of demyelination using cuprizone⁴ is important because it shows that normal animals can remyelinate after the drug has been removed from their diets. But this study does not explain the mechanism of remyelination, that is, whether the astroglial cells first withdraw their processes or whether the oligodendroglial cells displace them. In contrast to this model, most human demyelinating diseases³⁻⁵ exhibit an astroglial reaction but the degree of remyelination is poor to non-existent.

The earliest detectable morphological abnormality in this study of Jimpy mice lies in the astrocytes. To date, there is neither morphological nor biochemical evidence to suggest that the axons are abnormal at the onset of the astrogliosis. It remains to be deter-

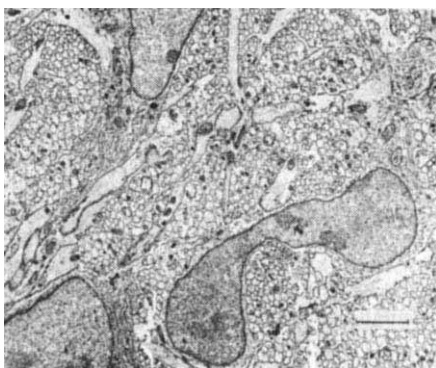


Fig. 1 8-d-old Jimpy mouse optic nerve exhibiting large groups of unmyelinated axons without contact to astroglial processes.

mined whether the astrogliosis is a reaction of the astrocyte to the axons or if it is inherent to the glial cell. As regards the relationship between the astrogliosis and myelination, the critical question is not so much the cause of the astrocytic abnormality but whether the condition itself interferes with myelination.

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¹ Meier, C. & Bischoff, A. *Nature* 267, 177 (1977).

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⁸ Oppenheimer, D. R. in *Greenfield's Neuropathology*,

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Nature of stromatoporoids

KAZMIERCZAK¹ argues that the spherulitic microstructure of stromatoporoids, together with the spherulitic infilling of astrorhizal canals in specimens described from the Upper Devonian of south-eastern Poland, indicate that they have been formed by the calcification of coccoid blue-green algal cells. This suggestion, that stromatoporoids are calcified cyanophyte colonies, is novel and stimulating but it is open to two principal objections. First, it fails to consider alternative explanations of the spherulites; secondly, it overlooks a variety of additional complex morphological features of stromatoporoids.

Stromatoporoids have also been compared with coelenterates^{2,3} and sponges⁴ and representatives of both groups have skeletons composed of aragonitic spherulites. The similarity of this to the granular or melanospheric microfabric of stromatoporoids has been detailed by Stearn⁵. Several non-skeletal origins of the spherule-like infillings of the astrorhizae described by Kazmierczak¹ are possible, including sedimentation of fine peloidal material, cementation, and recrystallisation. However, Kazmierczak does not deal with any of these points but instead assumes that calcification of coccoid cells must be the origin of skeleton and infilling alike.

The characteristic skeletal features of stromatoporoids such as laminae, pillars and canals together with the not uncommon cyst plates, basal layers and external epithelial coverings, appear to have no parallels in cyanophyte colonies. The permineralised cyanophyte colonies⁶ cited by Kazmierczak do not resemble stromatoporoids.

Kazmierczak is essentially proposing that stromatoporoids are skeletal stromatolites⁷ of the thrombotic variety⁸. Stromatoporoids and stroma-

tolites, although superficially similar, differ in external morphology, internal structure, and geological distribution. The complex and varied internal morphology and limited geological range of stromatoporoids, together with their tendency toward discrete forms, strongly suggest that they are higher organisms than cyanophytes.

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¹ Kazmierczak, J. *Nature* 264, 49–51 (1976).

² Lecompte, M. in *Treatise on Invertebrate Paleontology*. Part F (ed. Moore, R. C.) 107–144 (University of Kansas Press, Lawrence, 1956).

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Do antigen-specific helper factors in rabbit lack V-region Ig determinants?

TAUSSIG, Finch, and Kelus¹ proposed that antigen-specific helper factors derived from T cells in rabbit lack V-region Ig determinants. They further argued that T-cell surface receptor would also lack V-region Ig determinants since the V regions of both the helper factors and T-cell receptor would very likely be the same. Their proposal was based on the finding that sheep red blood cell (SRBC) specific helper factors derived from rabbit carrying an allotypic marker at the a locus in the V region of its immunoglobulins is not adsorbed by anti-allotype antibody (anti-al, Table 2 in ref. 1) conjugated to Sepharose. Since no adsorption occurred the authors interpreted it as an indication that the allotypic marker was not present in the SRBC-specific helper factors. In fact, Table 2 in ref. 1 shows that the helper activity is partly adsorbed by anti-al-Sepharose; the number of plaque forming colonies (PFCs) is reduced from $3,200 \pm 365$ to $2,450 \pm 280$ as a result of adsorption (Table 2, ref. 1). Since nothing is known about the relationship between PFC and quantity of helper factor used, it is difficult to say anything about the percentage of factor adsorbed by the immunoadsorbent. If the quantity of factor used in the control giving $3,200 \pm 365$ PFC is far greater than the optimum required (minimum quantity required to give maximum PFC) then the reduction in PFC after passing through the immunoadsorbent observed may be quite significant and represent a substantial reduction in helper activity. But, the authors have not studied the dependence of PFC on quantity of helper factor and not made any attempt to quantitate the factor before and after passing through the immunoadsorbent. It seems to me that the crude data presented do not

allow the proposal the authors have made.

If indeed the data show a lack of adsorption of the helper factor by anti-al-Sepharose, then the lack of adsorption can be explained in an alternate way also. Since the helper factor has the capacity to bind antigen and since the factor was generated by incubating lymphocytes with antigen, it is conceivable that the factor is complexed with antigen fragments. These antigen fragments, though non-immunogenic and incapable of sensitising the bone marrow cells, may be large enough to sterically block the allotypic (al) determinant and thus prevent recognition by anti-al antibody. The authors, thus, have not ruled out the possibility that V-region immunoglobulin determinants are present in antigen-specific helper factors and receptor molecules of T helper cells. Whether or not the antigen-recognising sites of T helper cell receptor molecules and the helper factors are controlled by immunoglobulin V genes in rabbit, thus, remains an open question.

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¹ Taussig, M. J., Finch, A. P. & Kelus, A. S. *Nature* 264, 776–778 (1976)

TAUSSIG *et al.* REPLY—Dr Sundharadas has raised certain points concerning removal of the rabbit antigen-specific T-cell factor on immunoadsorbents carrying anti-immunoglobulin (Ig) reagents. The difference in numbers of PFCs which he points out is not significant (at the 5% level). The capacity of the anti-al allotype adsorbent was more than sufficient, on past experience, to have removed the factor had the latter carried the al allotypic marker. As stated in the paper, the method was sensitive to a 50% reduction in factor activity, based on titration. The question is really not whether al-carrying molecules could have escaped detection, but rather whether a proportion of factor molecules greater than 50% could have Ig V regions yet be negative for an allotype, as are about 10% of normal rabbit Ig molecules. This cannot be ruled out, as discussed in our paper. Thus, at the very least our results show that the factor molecules do not carry the Ig allotype in the same proportion as circulating Ig. The further point about possible blocking of the factor binding site and steric hindrance of the allotype we consider unlikely. Evidence against it is that the factor can be absorbed by antigen, indicating the availability of its binding site.

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reviews

Basic modern astrophysics

Paul Davies

The Structure of the Universe. By Jayant Narlikar. Pp. 264. (Oxford University: London, Oxford and New York, 1977.) £4.25.

THE name of Jayant Narlikar has long been associated with the steady-state theory of the Universe, the absorber theory of radiation and other controversial aspects of modern cosmology. Now he has used his fertile imagination to produce a readable and useful introductory book to astronomy and cosmology. Although the first half of the book deals in a conventional manner with standard topics such as stellar evolution, galactic structure and Friedmann cosmological models, some of the legacies of the author's more speculative past research emerge in later sections. The chapter entitled *The Universe and the Arrow of Time* will be familiar to those who have read Professor Narlikar's earlier (much more specialist) book written with Sir Fred Hoyle, although the uninitiated will probably be unable to follow the subtleties of the arguments about past and future absorbers, and initiates may well disagree with the conclusions.

The text has obviously been prepared with a view to commanding a wide readership which can range from the scientifically-inclined layman seeking an easy-going summary of this popular subject, up to astronomy and physics undergraduates requiring background or introductory reading. To this end the mathematics has been kept to a minimum, and where possible is separated from the main part of the text in special boxes. There is a liberal distribution of graphs and line drawings, some of which seem to have been prepared hurriedly, and one of which (concerning the Friedmann cosmological models) is badly wrong and could be seriously misleading for a student. In spite of these slips, the open style and lucid presentation generally make information retrieval easy and straightforward.

Superficially, *The Structure of the Universe* seems like a popular book. Its jacket begins with the pregnant query "Do black holes really exist?", and follows up with such imponderables as "what is a quasar really like?" In spite of this, black holes only occupy

two or three pages buried in the chapter on gravitation, and most of the discussion about this and other much-exposed topics in modern astronomy is explanatory rather than descriptive.

There is an unmistakable philosophical flavour running through the discussion, with the theme that local physics and global structure are somehow interwoven and that recent developments in both observational and theoretical astronomy are beginning to provide a tantalising glimpse of this link. This is an idea certain to appeal to non-specialist readers who are frequently intrigued by the concept of a grand design, but students would do well to temper enthusiasm for this speculation

with healthy scepticism, for much of the physics of the 'micro-macro' link such as the absorber theory of radiation and Mach's principle, is not universally accepted. I was a little disappointed that Professor Narlikar, for so long a spirited advocate of the steady-state theory, has not taken the opportunity to present an extended counterattack in its favour.

In summary, this is a nicely written, attractive book, including many useful, if somewhat superficial, discussions of basic modern astrophysics, from an author of international repute. □

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Receptor research

Methods in Receptor Research, Parts 1 and 2. By M. Blecher. Pp. xiv+383 and 385-763. (Marcel Dekker: New York, 1976.) SFRs. 120 per part.

THESE two volumes have been designed to form a laboratory manual giving a more detailed description of methods used in receptor research than is commonly found in papers published in journals. In this aim, the editor and the 41 authors have succeeded. It was decided to omit the receptors for steroid and thyroid hormones because they have little in common with the polypeptide hormones, which take up most of the space. There are three chapters on gonadotropin receptors, three chapters dealing with glucagon receptors, three chapters covering insulin receptors, two chapters on prostaglandin receptors and one chapter each on β -adrenoceptor and on the receptors for acetylcholine, adrenocorticotropin, oxytocin, vasopressin, thyrotropin, parathormone, prolactin, cholera toxin, lectin and opiates. Each chapter is written by an expert in the field, describing the laboratory techniques in considerable detail. As with other treatises covering rapidly expanding areas of research, it could not be avoided that there are some notable absentees, as for instance the dopamine receptor and α -adrenoceptor.

The editor stresses in his preface that the overlap between several chapters

is more apparent than real. The reviewer finds himself in agreement with this view because only too often seemingly contradictory results obtained by different laboratories are due to minor but important differences in the experimental techniques used.

It is not possible to deal with the various chapters, which are all well and clearly presented. The newcomer to the field of receptor research will find the detailed description of many well tried methods useful. In addition, there are two carefully considered discussions of a more general nature: Catt *et al.* deal with problems encountered in the determination of binding constants and of concentration of receptor, whereas De Meyts presents sections dealing with the physico-chemical parameters determining optimal steady-state binding and its quantitative analysis, the factors of importance for the study of the kinetics of hormone-receptor binding, and the cooperative interactions among receptor sites.

These well produced volumes will be essential for laboratories engaged in receptor research. Unfortunately, the high price will prevent their acquisition by many individual workers.

H. W. Kosterlitz

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Cognitive development

Development of Cognitive Processes. Edited by Vernon Hamilton and Magdalen D. Vernon. Pp. 772. (Academic: London and New York, 1977.) £21.00.

It was not so long ago that childhood as a distinct state, with its own distinguishing qualities, was not part of the consciousness of our society. Children wore smaller versions of the same clothes as adults, did similar work, and were expected to behave as much like their elders as possible. There were hardly any books either for, or about, children. Judged by the present volume, although the rest of the world has in the meantime discovered childhood in an upsurge of kids' lib., developmental psychology is still in this undeveloped state.

Current psychological models of cognitive processing are based on empirical investigations with adults. In the present book these models seem to have been adopted without questioning their validity for child development. It is therefore not surprising, that most of the conclusions that are reached are of one of two kinds. First, children, from birth or from a very early age, may show certain information handling capacities, which differ little from those of adults. Alternatively, children are much worse than adults on many cognitive operations, though they become better as they get older. This view is expressed in statements such as: "Increasing age brings increasing ability to make cognitive decisions about a task, and to absorb more information"—which is perhaps not too surprising to anyone but developmental psychologists.

Some of the contributors are clearly aware of the shortcomings of this approach. Thus one of the editors urges in her conclusions that investigators should concentrate on the specific qualities and properties of cognitive processes in children. Two introductory, theoretical chapters do not even pretend to a relevance for development, and though in themselves adequate, state nothing which has not been published extensively elsewhere. In spite of the heavy reliance of cognitive models on the analysis of memory functions, which is reflected in these chapters, the book does not even contain an account of the development of memory.

As one might expect from such a miscellaneous collection, quality of style and content varies greatly. The first ranges from barely comprehensible jargon, such as: "It is worth restating,

therefore, that human information processing systems may be variably aroused by diverse mechanisms and cognitive events, that they contain dynamically organised goal-seeking programmes, and that they possess an autonomous general capacity and need to continue operations upon inputs and their stores, which elaborate and reorganise their representations long after the event", to the admirable simplicity of statements such as: "We have two eyes and the most natural way to perceive space is through both of them".

There are excellent accounts of perception of form by Vernon, spatial perception by Walk, conceptualisation

by Donaldson and language by Cromer. But in spite of the high qualities of these contributions, and notwithstanding the irrelevant, pseudoscientific design of the dust jacket, there is little indication in this book that developmental psychology has begun to put forward its own models and theories. These will have to account for the specific qualitative rather than the merely quantitative characteristics of children's cognitive processes.

In the meantime, *vivat vivat* Piaget!

Beate Hermelin

Beate Hermelin is a Research Psychologist in the MRC Developmental Psychology Unit, London, UK.

Plant embryogenesis

Experimental Embryogenesis in Vascular Plants. By V. Raghavan. Pp. 603. Academic: London and New York, 1977.) £21.00, \$45.65.

In an era of symposia and other multi-authored volumes, V. Raghavan deserves to be congratulated on producing this fine volume dealing with a very complex subject which has an immense literature. The reader is immediately impressed with the breadth of topics and the depth of treatment that most receive.

The first and longest section of the book deals with the development from egg to embryo. Among the topics covered by chapters are: structure and organisation of the egg, zygote and embryo; biochemical embryogenesis; nutritional aspects of embryogenesis—both physiological and morphological considerations; embryo culture; embryogenesis in cultured ovaries, ovules and seeds; nutrition and metabolism of cultured embryos; extracellular control of morphogenesis; biochemical ontogeny of cultured embryos, and applied aspects of embryo culture. I was especially impressed with this section because of the excellent organisation, lucid writing and relating of various approaches to one another. While most of the information deals with angiosperm embryos, references are made to pteridophyte and gymnosperm embryos when appropriate.

The second section discusses adventive embryogenesis, including the induction of both haploid and diploid embryoids. The author has made these chapters very informative, for he has not merely summarised the literature but has tried to make some sense out

of the many factors controlling embryoid formation, showing clearly that more work is necessary before these factors are understood satisfactorily. A similar appeal is made in relation to the low percentage of microspores that give rise to haploid embryoids. The last section deals with seed dormancy and germination and no doubt many readers will disagree with the author's decision to include them. The last chapters, however, are useful in giving a full perspective of embryogenesis. The argument is put forth that dormancy involves a mechanism of preventing embryo growth and that factors regulating the normal growth and development of the embryo should also apply to the induction and release of dormancy. All three sections are written with clarity and usefully illustrated with drawings, micrographs and graphs. Unfortunately some of the electron micrographs are printed too darkly but on the whole the book's illustrations are very informative. An appendix containing the mineral salt composition of 18 different nutrient media used in embryo culture should prove useful for many readers. The 103 pages of references should also be valuable to the reader.

In the introduction Professor Raghavan expresses the hope that the book will bring together some of the scattered embryological literature and present the current and future perspectives of plant embryogenesis. The book not only attains that goal in its comprehensive treatment but undoubtedly this book will be the definitive volume on experimental plant embryogenesis for some time. It is highly recommended for all those interested in this most interesting and important area of plant morphogenesis.

William Newcomb

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Magnetic ions in metals

Magnetic Ions in Metals: A Review of their Study by Electron Spin Resonance. By R. H. Taylor. Pp.+118 (Taylor and Francis: London, 1977.) £6.

THE first observation of electric spin resonance (ESR) was reported by Zavoisky in 1945. Since that time the ESR spectrum of every transition and rare earth ion has been observed in non-metallic solids in one valence state or another. The ESR spectrum is usually studied in single crystals to elucidate interactions between the magnetic ion and its local environment. As a result almost all features of these spectra are understood at least in terms of a phenomenological spin Hamiltonian. There have been many reviews of the properties of magnetic ions in non-metal host lattices: the important results and theory are collected in the encyclopaedic monograph of Abragam and Bleaney (1970). No similar authoritative volume has appeared on magnetic

ions in metals largely because the application of ESR to metals developed rather slowly; thus most of the important work has been done since 1970. The history of this development is well compiled by Dr Taylor in this welcome addition to the literature on magnetic resonance.

Reasons for the tardy growth in this particular area are to some extent lost to non-experts on metals, but metallurgical factors including single crystal growth have been especially important. I would hardly agree with the author that the rather dramatic arousal of interest since about 1970 is attributable to "increasing efficiency of spectrometer systems" or to "increase in spectrometer resolution", both experimental factors which have changed little since 1962. This book is conveniently divided into three major parts. After some introductory remarks, Dr Taylor gives a very good summary of the main applications of the ESR method to metals: studies of ground state splittings, the ESR bottleneck, exchange interactions of magnetic ions with conduction electrons, magnetic order in concentrated alloys, magnetic ions in type II superconductors are all discussed. But by far the major part of the monograph is concerned with

an experimental review of the spectra of magnetic ions in simple metals, binary alloys and intermetallic compounds. It may come as a surprise to some readers how few ions with well characterised spectra have been observed in metal host lattices. Most published work concerns the S-state ions Mn, Fe, Gd and Eu; far less has been achieved with non S-state ions, only Er, Dy, Yb and Ce having been observed at all. The monograph is completed by a most valuable review of possible growth areas for ESR studies of metallic solids.

The major strength of the book is that it says clearly what has been achieved up to 1975. It could have been improved somewhat by a more substantial discussion of both the folklore of the subject (for example, what systems have been tried but found not to work) and the theoretical background. But in all it is a well produced volume which will be a valuable addition to the bookshelf of both workers in the field and physicists more generally interested in magnetic resonance.

B. Henderson

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Microbiological aspects of waste treatment

Treatment of Industrial Effluents. Edited by A. G. Gallely, C. F. Forster and D. A. Stafford. Pp. 378 (Hodder and Stoughton: London, Sydney, Auckland and Toronto, 1977.) £7.95.

THIS book combines the work of twenty-two authors in twenty-one chapters covering aspects of waste treatment from general description of the basic processes to the problems and treatment of specific industrial wastes. The depth of treatment varies from chapter to chapter, as might be expected with such a large number of authors. Some topics are dealt with in considerable detail and the authors in these cases quote numerous references; the chapter on Surfactant Biodegradation in Waste Waters is particularly valuable.

The editors should have avoided such obvious mistakes as on page one, where the statement is made that "activated sludge plants began to be operated by 1900 AD", when a later author in chapter 5 gives the correct date for the development of the process in 1914. There is also no reason in

chapter five to substitute the term 'trickle filters' for the professionally accepted terms of either 'trickling filters' or 'biological filtration'. In chapter 4 it is difficult to interpret the phrase in the section on the determination of nitrate and nitrite; "clean waste such as potable supplied".

The title of the book would have been more explicit if it had included the word 'microbiological'. The book is heavily biased to the microbiological aspects of waste treatment, and it would have been improved by including better information on the physical and physicochemical aspects of waste treatment and an introduction to the modern concepts of mathematical description of the processes.

Despite these criticisms, the book will be particularly useful to post-graduate students working in the field.

The amount of information presented in the book, which includes over 800 references, represents a very useful addition to the British textbooks available. The book is well produced and very readable and, compared to other similar books, is good value at the price.

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Agricultural research strategy

Resource Allocation and Productivity in National and International Agricultural Research. Edited by T. M. Arndt, D. G. Dalrymple and V. W. Ruttan. Pp. 617. (University of Minnesota Press: Minneapolis, 1977.) \$25.

CAN science be justified in quantitative economic terms? If no convincing reply, one way or the other, has yet been given, some progress has been made towards the economic analysis of one area, namely research carried out to create new agricultural technology. This research is mostly publicly funded and so has identifiable expenditures; moreover, it generates evident benefits, such as increases in crop yields, which can be quantified. It is virtually indisputable that increases in food supply must improve the welfare of mankind.

Griliches's pioneering study of hybrid maize first provided a methodology, and by 1969 it was possible to hold a symposium on *Resource Allocation in Agricultural Research* (ed. W. L. Fishel, University of Minnesota Press, 1971). We now have the Airlie House conference, which takes account of developments since then.

The book consists of 29 papers which were re-written, and edited, after the symposium. This has blunted the sharp edge of controversy, and it might have been interesting to have included one review paper by a devil's advocate. An issue he might have pursued is the gap which exists between theoreticians (especially model builders) and those needing practical techniques.

Even with careful editing, it could hardly be expected that a coherent synthesis of the whole field would emerge, but the papers provide valuable background on several particular aspects. These are: descriptions of agricultural research institutions, especially those for developing countries, economic analysis of national and international agricultural research systems, and methods of planning and organising agricultural research. It may be mentioned that a good account of the conference is available free as a seminar report from the Agricultural Development Council, New York.

Although all the social implications of technical change in agriculture could not be fully explored, the conference has importance as a deliberate attempt at closer collaboration between social and other scientists, an initiative which is to be welcomed.

The main theme was the "social rate of return on agricultural research", a concept deriving from Griliches. It is well known, even notorious, that such calculations lead to very high numbers, but this raises some queries. Why is agricultural research so productive? If so, why do governments not invest more in it? And if subjective planning methods have proved so successful, why seek to replace them by more elaborate, but still untried, methods?

Although a consensus at the conference did not accept it, the suggestion was made that the rate of return is an inappropriate planning indicator, since it implies that there are no constraints on other factors such as land, labour, energy, capital, imports, and so on. Viewed thus, the high rates of return are only artefacts, and the way

could then be open for a different and more realistic presentation to governments of the important contribution which agricultural research can make to national economies.

It is possible, therefore, that the Airlie House conference will prove to be a watershed. Griliches's original study has been prolific, but we need to subject the methodology to close scrutiny and to consider developing improved procedures. This could well be the theme for another conference; meanwhile, this volume provides economists and those concerned in agricultural research strategy with a useful source on current thinking.

W. S. Wise

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Free-living and parasitic Protozoa

Protozoa. By A. Westphal (in collaboration with H. Muhlphardt). (Translated from the German by W. H. R. Lumsden and G. A. Targett.) Pp. 325. (Blackie: Glasgow and London, 1976.) £9.90.

WITH a few notable exceptions one rarely finds modern texts which encompass both free-living and parasitic Protozoa. Any new publications in the field are therefore welcomed. This is a book of high technical quality. It is profusely illustrated with delightful line drawings which, combined with a use of clear and varied typeface in the text, produces a book of immediate visual impact.

From the outset the author has imposed well defined limits on the objectives of this book. In the preface he states "it is not intended to be, and cannot be, anything more than an introduction to protozoology". To this end, the book is divided into three sections: the first, which occupies half the text, deals with the taxonomy of the phylum; the second describes the myriad cell organelles found in these organisms; the final section briefly discusses the ecology of the Protozoa.

In the taxonomic section the wealth of illustration excels. Each class is described in the text concisely, although frequently at the expense of clarity. The description is followed by diagrams showing the range of morphology and life cycles in the relevant organisms. This format does however become wearing on the reader when the illustrations are referred to frequently in the text. The inclusion of a systematic key

would have increased the practical value of this book.

The description of the cell biology of the Protozoa provides an insight into the amazing complexity of the sub-cellular morphology achieved in this 'simple' group of organisms. It is unfortunate that the reproduction of the electron micrographs does not match the quality of the line diagrams. Here, as elsewhere in the book, there is a dogmatic tenacity for a scientifically accurate text rather than a simple and readable one. It is inevitable in such a wide ranging introductory book that that author's necessity for generalisation introduces errors in specific instances. Moreover, the description of meiosis and mitosis does not match the standard set by the remainder of the text, and could well have been omitted.

The final chapter of the book, on the Protozoa and their environment, briefly examines the distribution of some common free-living Protozoa and provides a more expansive and highly readable section of the impact of the principle protozoan parasites of man and his domestic animals.

It is inevitable that this not inexpensive book be compared with Grell's *Protozoology*, a book of similar technical layout and scientific interest, which, although much more expensive, covers all aspects in great depth. Although the latter will certainly be the choice of the committed protozoologist, Westphal's text is one that could be considered for many undergraduate applications.

R. E. Sinden

R. E. Sinden is Lecturer in Zoology in the Department of Zoology and Applied Entomology at Imperial College Field Station, Ascot, UK. He is Secretary of the British Section of the Society of Protozoology.

announcements

Appointments

Dr J. H. Steele, to Director of the Woods Hole Oceanographic Institution, October 1977, from his position as Deputy Director of the Marine Laboratory of Agriculture and Fisheries for Scotland.

Dr D. Silverleaf, to Director of Resource Planning, a new post in the Central Electricity Board's recently formed Research Division, from Director of Resource Planning in the South Western Region.

Awards

The French Society of Physics has granted its awards for 1977. The Jean-Ricard Physics Award was received by Roger Balian for his work at the Saclay Centre for Nuclear Studies (Commission for Atomic Energy). Roger Balian is a theoretician of many particle systems and has worked on various subjects from nuclear physics to the physics of condensed matter. He also did research on helium 3 and wave behaviour.

The Jean-Perrin Award for the Popularisation of Science was shared by Robert Clarke and Nicolas Skrotzky from France-Culture.

The Robin Award, generally granted to a physicist for the whole of his work, went to M. P. Cagnac, a specialist in atomic physics teaching at the Paris VI University.

American scientists Seymour Benzer, a biologist, and John Dyson, a physicist, are the 1977 recipients of the Harvey Prize. Seymour Benzer, Professor of Biology at the California Institute of Technology, receives the Harvey Prize in Human Health for his discoveries in molecular and behavioural genetics, and John Dyson, Professor of Physics at the Institute of Advanced Studies, Princeton, receives the Harvey Prize in Science and Technology for his work in the fields of quantum electrodynamics, ferromagnetism, field theory, static mechanics and the stability of matter.

The Institute of Physics, London, and the German Physical Society announce the award of the 1977 Max Born Medal and Prize to Professor W. E. Spear of Dundee University, for his work on charge transport in non-crystalline semiconductors.

The Biochemical Analysis Prize (10,000 DM) donated every two years by Boehringer, Mannheim, BRD, for outstanding work in the field of biochemical instrumentation and analysis. The presentation will take place during the 1978 Analytical Biochemistry conference in Munich from 18 to 21 April. One or several papers concerning one theme, either published or accepted for publication between 1 October 1975 and 30 September 1977, may be sent in triplicate to Professor I. Trautschold, Secretary of the Biochemical Analysis Prize, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, 3000 Hannover 61, BRD, by 15 November 1977.

Person to Person

Exchange 3-bedroom house in Mill Valley, Marin County, California, for similar in north Oxford or nearby for one year beginning August/September 1977. Easy commute to San Francisco. Contact David Martin, Jr, Department of Medicine, University of California, San Francisco, California 94143.

Exchange 3-bedroom house easy access Adelaide, Australia, for similar in London area, December 1977 to January 1979. Contact A. Grady, School of Earth Sciences, Flinders University of S.A., Bedford Park, South Australia 5042.

There will be no charge for this service. Send items (not more than 60 words) to Marcus Dobbs at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

Meetings

18-20 July, **1977 Summer Computer Simulation Conference**, Chicago, Illinois (G. Graber, Treasurer 1977 SCSC, Applied Dynamics International Inc., 3800 Stone School Road, Ann Arbor, Michigan 48104, USA).

7-12 August, **1st International Congress for the Study of Child Language**, Tokyo, Japan (F. C. C. Peng, International Christian University, 10-2, 3 Chome, Osawa, Mitaka, Tokyo 181, Japan).

23-24 July, **4th ICU Symposium on Sociolinguistics**, Tokyo, Japan (Department of Linguistics, International Christian University, 10-2, 3 Chome, Osawa, Mitaka, Tokyo 181 Tokyo, Japan).

30-31 July, **2nd International Symposium on Pedolinguistics**, Tokyo, Japan (Department of Linguistics, International Christian University, 10-2, 3 Chome, Osawa, Mitaka, Tokyo 181, Japan).

25-28 August, **The Linderstrom-Lang Conference 1977 on Biochemical Control Mechanisms of Respiration at the Molecular and Tissue Level**, Espoo, Finland (Department of Medical Chemistry, University of Helsinki, Sittavuorenpenker 10, SF-00170 Helsinki 17, Finland).

25-30 August, **International Conference on Chemical Education**, Ljubljana, Yugoslavia (International Union of Pure and Applied Chemistry, University of Ljubljana, Department of Chemistry, RCPU, PO Box 18/1, 61001 Ljubljana, Yugoslavia).

26-30 August, **85th Annual Convention of the American Psychological Association**, San Francisco (Information Office, American Psychological Association, 1200 Seventeenth Street, N.W., Washington, DC 20036, USA).

4-17 September, **Training Course on Membrane Biophysics and Biochemistry**, Cambridge, UK (V. L. Lew, Physiological Laboratory, Downing Street, Cambridge, UK).

8-9 September, **Conference on The Mechanics of Granular Materials**, London, UK (The Institute of Mathematics and its Applications, Maitland House, Warrior Square, Southend-on-Sea, Essex, UK).

11-24 September, **Summer School—Thermal Neutron Scattering**, Harwell, UK (B. T. M. Willis, MPD/521.2, AERE Harwell, Didcot, Oxon, UK).

19-21 September, **Biennial Polymer Physics Conference**, Shrivenham, UK (Institute of Physics, 47 Belgrave Square, London SW1, UK).

26-29 September, **1st BOC Priestly Conference on Heterogeneous Oxidation**, Leeds, UK (J. T. Cleave, Special Courses Division, Department of Adult Education and Extramural Studies, University of Leeds, Leeds, UK).

26-29 September, **6th Europhysics Conference on Macromolecular Physics, Phase Transitions in Bulk Polymers**, Varna, Bulgaria (M. Mihailov, Bulgarian Academy of Sciences, Central Laboratory for Polymers, EPS Conference, 1113 Sofia, Bulgaria).

29 September, **Symposium on Food Contamination**, Manchester, UK (G. A. Barker, CPC (United Kingdom) Ltd, Trafford Park, Manchester 17, UK).

Reports and Publications

UK & Ireland—May

- Science Policy Research Unit. Annual Report 1976. Pp. 68. (Falmer, Brighton: SPRU, University of Sussex, 1977.) [25]
- The British Institute of Radiology. Annual Report and Accounts, 1976. Pp. 16. (London: The British Institute of Radiology, 32 Welbeck Street, W1, 1977.) [25]
- Medical Research Council. Review of Dental Research: a Report by the MRC Dental Committee. Pp. 58. (London: MRC, 20 Park Crescent, W1, 1977.) [25]
- National Radiological Protection Board. NRPB-R58: A Revised Oceanographic Model to Calculate the Limiting Capacity of the Ocean to Accept Radioactive Waste. By G. A. M. Webb and P. D. Grimwood. Pp. 19. (Harwell, Didcot, Oxon: National Radiological Protection Board, 1976.) [25]
- Conservation and Metallurgical Process Design. By Herbert H. Kellogg. (Thirteenth Sir Julius Wernher Memorial Lecture of the Institution of Mining and Metallurgy.) Pp. 11. (London: The Institution of Mining and Metallurgy, 44 Portland Place, W1, 1977.) [25]
- The Welfare of Laboratory Animals: Legal, Scientific and Humane Requirements. (Proceedings of a Symposium organized by The Universities Federation for Animal Welfare, The British Laboratory Animals Veterinary Association, and the Laboratory Animal Science Association, 30th September and 1st October, 1976.) Pp. 131. (Potters Bar, Herts: The Universities Federation for Animal Welfare, 1977.) [45]
- World Energy Conference. Symposium—Conservation Commission Review and African Energy Prospects, held in Abidjan, Ivory Coast on the 12th October, 1976, on the occasion of the meeting of the International Executive Council. Pp. 84. (London: World Energy Conference, 34 St. James's Street, SW1, 1977.) [45]
- European Brewery Convention. Report of their Barley Committee: Field Trials 1972 (Malting 1973). Pp. ii + 284. (Cambridge: National Institute of Agricultural Botany, 1977.) [55]
- Philosophical Transactions of the Royal Society of London. B: Biological Sciences. Vol. 278, No. 962: A Discussion on Resource Development in Semi-Arid Lands. Organized by Sir Josephy Hutchinson, FRS., A. H. Bunting, A. R. Jolly and H. C. Pereira, FRS. Pp. 437-6144 2 plates. (London: The Royal Society, 1977.) UK £10.60; Overseas £11.10. [55]
- Department of the Environment. Government Response to the Yorkshire and Humberside Regional Strategy Review 1975—The Next Ten Years. Pp. 19. (London: Department of the Environment, 2 Marsham Street, SW1, 1977.) [65]
- No. 10 Downing Street. (Department of the Environment.) Pp. 12. (London: HMSO, 1977.) 50p net.
- British National Bibliography. Research Fund Report No. 1: The Reading Habits of Adults—a Select Annotated Bibliography by Margaret Mann. Pp. 72. (London: Publications Officer, British Library Research and Development Department, Sheraton House, Gt. Chapel Street, W1, 1977.) £5.95 (cash with order). [95]
- Philosophical Transactions of the Royal Society of London. A: Mathematical and Physical Sciences. Vol. 285, No. 1327: The Moon—a New Appraisal from Space Missions and Laboratory Analyses. Pp. 1-606. (London: The Royal Society, 1977.) UK £36.50; Overseas £37.50. [95]
- Tetrahedron Reports. No. 20: The Molecules $R_2C=CR_2$ Including Azomethine, Carbonyl and Thio-carbonyl Ylides. Their Syntheses, Properties and Reactions. By Richard M. Kellogg. Pp. 20. £4.95. No. 21: Synthetic Uses of Anodic Substitution Reactions. By Lennart Ebersson and Klas Nyberg. Pp. 22. £4.95. No. 22: An Empirical Analysis of the Circular Dichroism of Chiral Olefins. By John Hudec and David N. Kirk. Pp. 32. £4.95. No. 23: Structure and Reactivity of Cycloimmonium Ylides. By G. Surpateanu, J. P. Cateau, P. Karafiloglou and A. Labache-Combiere. Pp. 17. £4.95. No. 24: The Mechanism of Epoxidation of Olefins by Peroxides. By V. G. Dryuk. Pp. 12. £4.95. No. 25: Ketone Enolates: Regiospecific Preparation and Synthetic Uses. By Jean d'Angelo. Pp. 12. £4.95. (Oxford and New York: Pergamon Press, 1977.) [105]
- Broadcasting to a Community in Conflict—the Experience in Northern Ireland. By Richard Francis. (Lecture given at the Royal Institute of International Affairs, Chatham House, 22 February 1977.) Pp. 16. (London: BBC, 1977.) [115]
- Department of the Environment. Environmental Standards: a Description of United Kingdom Practice. (The Report of an Inter-Departmental Working Party. Pollution Paper No. 11.) Pp. v + 30. (London: HMSO, 1977.) 75p net. [125]
- Department of Health and Social Security. Health and Personal Social Services Statistics for England (with summary tables for Great Britain), 1976. Pp. 200. (London: HMSO, 1977.) £5.50 net. [135]
- Mazingira, the World Forum for Environment and Development. No. 1, 1977. Published quarterly. Pp. 1-98. Annual Subscription: \$10; Single copies \$2.50. (Oxford: Robert Maxwell, Pergamon Press, 1977.) [135]
- Evaluation in Education: International Progress, Vol. 1, No. 1, 1977. Edited by Dr. Bruce H. Choppin and Dr. T. Neville Postlethwaite. Pp. 1-72. (Oxford and New York: Pergamon Press, 1977.) £4.50. [135]
- Mullard Research Laboratories. Annual Review 1976. Pp. 52. (Redhill, Surrey: Mullard Research Laboratories, Cross Oak Lane, 1977.) [135]
- Nuclear Power and the Environment. (Comments on the Royal Commission Report on Environmental

- Pollution.) Pp. 16. (London: The Institution of Professional Civil Servants, Northumberland Street, WC2, 1977.) [135]
- Proceedings of the Royal Irish Academy. Section A: Mathematical and Physical Sciences. Vol. 77, A, No. 1: Asymptotic Linearity and Nonlinear Eigenvalue Problems II. By J. F. Toland. Pp. 1-12. 96p. Vol. 77, A, No. 2: Diffusion Equation Study of Rotational Brownian Motion. By J. McConnell. Pp. 13-30. £1.80. Section B: Biological, Geological and Chemical Sciences. Vol. 77, B, No. 2: A Re-Appraisal of Some Ordovician Successions in Eastern Ireland. By P. J. Branchley, J. C. Harper, W. I. Mitchell and M. Romano. Pp. 65-85 + 1 plate. £1.23. Vol. 77, B, No. 3: A Comparison between *In situ* Photosynthetic Rates Determined Using ^{14}C Uptake and Oxygen Evolution Methods in Lough Neagh, Northern Ireland. By D. H. Jewson. Pp. 87-99. 69p. (Dublin: Royal Irish Academy, 1977.) [165]
- Trade Literature: a Review and Survey. By Martin J. Thomson. Pp. 52. (London: British Library, Science Reference Library, 1977.) £3. [165]
- A Key to the Adults and Nymphs of the British Stoneflies (Plecoptera), with Notes on Their Ecology and Distribution. By Dr. H. B. N. Hynes. Pp. 90. Third edition. £1. A Key to the Larvae and Adults of British Freshwater Megaloptera and Neuroptera, with Notes on Their Life Cycles and Ecology. By J. M. Elliott. Pp. 51. £1. (Ambleside, Cumbria: Freshwater Biological Association, The Ferry House, 1977.) [165]
- The Institution of Chemical Engineers. The Association of Cost Engineers. A New Guide to Capital Cost Estimating. Pp. 76. (Rugby: Institution of Chemical Engineers, George E. David Bldg., 165/171 Railway Terrace, 1977.) £3. [165]
- Brown Boveri Kent Limited. Report and Accounts 9 Months ended December 31, 1976. Pp. 23. (Luton: Brown Boveri Kent Limited, 1977.) [165]
- Rat News Letter. No. 1, April 1977. Pp. 73. (Carshalton, Surrey: Medical Research Council, Laboratory Animals Centre, Woodmansterne Road, 1977.) [165]
- Ministry of Overseas Development. Annual Report of the Directorate of Overseas Surveys for the year ended 31st March 1976. Pp. 59 + 8 plates. (London: HMSO, 1977.) £3.50 net. [175]
- The 1977-1978 Aldrich Catalogue Handbook of Organic and Biochemicals. Pp. 999. (Gillingham, Dorset: Aldrich Chemical Co., Ltd., The Old Brickyard, New Road, 1977.) [185]
- Department of Industry. Report on Research and Development, 1974-1976. Pp. 60. (London: HMSO, 1977.) £1.25 net. [185]
- Philosophical Transactions of the Royal Society of London. A: Mathematical and Physical Sciences. Vol. 284, No. 1326: A Discussion on Methods and Applications of Ranging to Artificial Satellites and the Moon. Organized by Alan H. Cook, FRS, D. G. King-Hele, FRS, S. A. Ramsden and A. R. Robbins. Pp. 419-623 + 2 plates. UK £11.90; Overseas £12.40. Vol. 285, No. 1328: On Linear Wave Motions in Magnetic-Velocity Shears. By I. A. Eltayeb. Pp. 607-636. UK £1.80; Overseas £1.85. (London: The Royal Society, 1977.) [185]
- Advisory Committee on Oil Pollution of the Sea—Annual Report 1976. Pp. 36. (London: Advisory Committee on Oil Pollution of the Sea, 39 Victoria Street, SW1, 1977.) [195]
- The Cotton Silk and Man-Made Fibres Research Association. Shirley Institute Report and Accounts, 1975-76. Pp. 24. (Didsbury, Manchester: Shirley Institute, 1977.) [195]
- Reading University Computer-Based Packages for Teaching Earth Science Report No. 3: Computer Programs in BASIC for the Interpretation of Gravity, Magnetic and Resistivity Profiles. By C. McCann (with Programs by S. Watts). Pp. 100. (Geological Reports, No. 9). £1.50. Report No. 4: The Computation and Display of Geotechnical Properties for Sediment Cores Using BASIC and FORTRAN. By Andrew Parker and Roger Till (with programs by Margaret Moss). Pp. 63. (Geological Report No. 10). 75p. Report No. 6: Programs in BASIC for Non-Linear and Multivariate Least-Squares Methods (with Worked Examples). By Roger Till (with programs by Duncan Cowan, Margaret Moss and Sue Watts). Pp. 87. (Geological Reports No. 12.) £1.50. (Reading: Geology Department, The University, 1977.) [195]
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- Office of Population Censuses and Surveys: Medical Statistics Division. Mortality Surveillance 1968-1975. England, Wales, Death and Rates by A-list Cause, Sex and Age Group. Pp. 150. (Fareham, Hampshire: Office of Population Censuses and Surveys, 1977.) Individual Analysis sheets 124p each (plus VAT p & p 10p). Spirally bound complete set £14.50 (plus VAT + p & p 50p). Microfilm version of complete set £2.50 (plus VAT + p & p 20p.) [205]
- National Radiological Protection Board. Annual Research and Development Report 1976. Pp. 152. (Harwell, Didcot, Oxon: National Radiological Protection Board, 1977. On sale through HMSO.) £1.75. [205]
- The Institution of Gas Engineers. Communication 1018 Domestic Gas in an Energy Conscious Future. By J. A. Demont, S. R. Kirk and E.A.K. Patrick. Pp. 27. £125. Communication 1019: The Planning and Management of Conversion. By B. C. Smith. Pp. 33. £1.25. Communication 1020: Preaching to the Unconverted. By J. A. McCawley. Pp. 22. £1.25. Communication 1021: Conversion in Retrospect—The Contractor's Point of View. By W. Richardson. Pp. 21. £1.25. Communication 1022: Some Recollections of the Technical Aspects

- of Conversion. By R. W. Byford, A. E. Sharman and J. Shuttleworth. Pp. 51. £1.25. Communication 1023: The Conversion of Central London, II. By F. A. Collins and J. D. Greene. Pp. 28. Communication 1024: Engineering Management in Engas. By C. H. Brown. Pp. 38. Communication 1025: Computer Based Customer Systems. By P. G. Clarke and R. Gofford. Pp. 24. £1.25. Communication 1026: Text Processing—Towards a Paperless Office. By J. E. Hogben and B. A. Izzard. Pp. 21. £1.25. Communication 1027: Quality Assurance—Two British Gas Approaches. By J. Cairns and J. Boddy. Pp. 24. £1.25. Communication 1028: Telling is Not Communicating. By G. Bostock. Pp. 14. £1.25. Communication 1029: 'Putting it Across' to Factory Managers, Plant Engineers, Local Authorities, etc. By P. W. King. Pp. 13. £1.25. (114th Annual General Meeting, Bournemouth, May 1977.) (London: The Institution of Gas Engineers, 17 Grosvenor Crescent, SW1, 1977.) [235]
- Mathilda and Terence Kennedy Institute of Rheumatology. Report for 1976. Pp. 31. (London: Mathilda and Kennedy Institute of Rheumatology, 6 Bute Gardens, W6, 1977.) [235]
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- Philosophical Transactions of the Royal Society of London. B: Biological Sciences. Vol. 277, No. 955: A Discussion of the Meiotic Process. Organized by R. Riley, FRS, M. D. Bennett and R. B. Flavell. Pp. 183-376 + 28 plates. (London: The Royal Society, 1977.) UK £17.05; Overseas £17.60. [235]
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- AERE, Harwell. AERE-R 8671: Radioactive Fallout in Air and Rain—Results to the End of 1976. By R. S. Cambray, Miss E. M. R. Fisher, J. D. Eakins and D. H. Peirson. Pp. 42. (London: HMSO, 1977.) £1.50 net. [245]
- Pharmaceutical Industry Press Directory. Pp. 24. (London: The Association of the British Pharmaceutical Industry, 162 Regent Street, W1, 1977.) [255]
- Fruits and Vegetables in Particular: Rita Greer's Second Kitchen Notebook. Pp. 106. (London: Roberts Publications, 225 Putney Bridge Road, SW15; Portsmouth: Rita Greer, Wallisdean Avenue, 1977.) £2.30. [255]
- East Malling Research Station. Report for 1976, 1st October 1975 to 30th September 1976. (The Kent Incorporated Association for Promoting Experiments in Horticulture.) Pp. 220. (Maidstone, Kent: East Malling Research Station.) £2.50; \$5. [305]
- Review Body on Doctors' and Dentists' Remuneration—Seventh Report, 1977 (Cmnd. 6800). Pp. v + 72. (London: HMSO, 1977.) £1.35 net. [305]
- Common Waiting List for NHS and Private Patients in NHS Hospitals. (Report by the Health Services Board made under Section 6 of the Health Services Act 1976.) (Cmnd. 6828) Pp. 15. (London: HMSO, 1977) 35p net. [305]
- Philosophical Transactions of the Royal Society of London. B: Biological Sciences. Vol. 279, No. 963: A Discussion of Scientific Research in Antarctica. Organized by Sir Vivian Fuchs, FRS, and R. M. Laws. Pp. 1-288 + 8 plates. (London: The Royal Society, 1977.) UK £18; Overseas £18.50. [305]
- Report to the Worshipful Company of Clothworkers of the City of London of the Advisory Committee on the Departments of Textile Industries and Colour Chemistry and Dyeing in the University of Leeds for the Session 1975-1976. Pp. 60. (Leeds: The University, 1977.) [315]
- ## Other countries—May
- The Zoological Institute, Faculty of Science, University of Tokyo. Annual Report for 1976. Pp. 31. (Tokyo: The University, 1977.) [25]
- The Center for the Study of Drug Development, University of Rochester Medical Centre, Rochester, New York. Recurrent Criticisms: a History of Investigations of the FDA. By Jeanne Herzog. Pp. 9. Some Homework is Needed. By Alexander M. Schmidt. Pp. 7. (Rochester, New York: The Center for the Study of Drug Development, 1977.) [25]
- United States Geological Survey. Annual Report, Fiscal Year 1976. Pp. iii + 212. (Washington, DC: US Government Printing Office, 1977.) [25]
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- United States Department of the Interior: Geological Survey. Bulletin 1421-C: The Volumetric Properties of Aqueous Sodium Chloride Solutions from 0°C to 500°C at pressures up to 2,000 Bars Based on a Regression of Available Data in the Literature. By Robert W. Potter II and David L. Brown. Pp. iii + 36. (Washington, DC: US Government Printing Office, 1977.) [45]
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